Investigation of the Solubility and Enzymatic Activity of a Thioredoxin-Gelonin Fusion Protein

by

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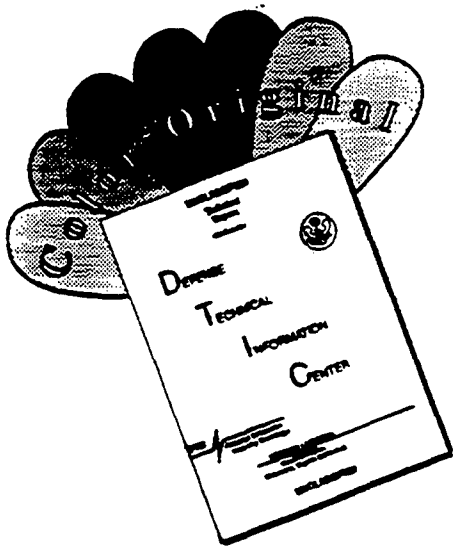

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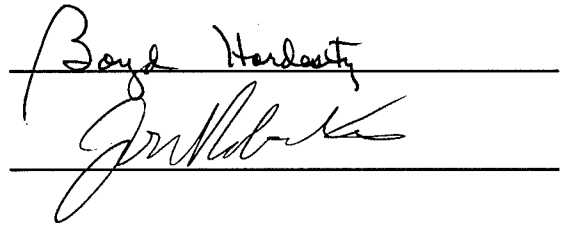

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Investigation of the Solubility and Enzymatic Activity of a Thioredoxin-Gelonin Fusion Protein

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The University of Texas at Austin, 1997

Supervisor: Boyd A. Hardesty

A synthetic gene for the ribosome-inactivating protein (RIP), gelonin, was previously engineered and inserted into the pET-21a plasmid under the control of the T7 promoter by researchers at M.D. Anderson Cancer Research Institute in Houston, Texas. Upon induction of *Escherichia coli* (*E. coli*) strain BL-21(DE3)pLysS containing this pET-21a/gel plasmid, the resulting gelonin protein forms insoluble aggregates, known as inclusion bodies, and exhibits no activity under the assay conditions tested. By genetically fusing gelonin to the highly stable and soluble protein, thioredoxin, it was thought that there would be an increase in the solubility of gelonin, possibly accompanied by a measurable amount of enzymatic activity.

In this research, a plasmid (pET-32a/gel) containing the genetic information to synthesize a thioredoxin-gelonin fusion protein was engineered. The protein was overexpressed in *E. coli* strain BL-21(DE3)pLysS and also synthesized by *in vitro* coupled transcription/translation. The solubility of thioredoxin-gelonin was not

increased compared to gelonin when overexpressed in *E. coli*, but was increased from 22% to 71% when synthesized by *in vitro* coupled transcription/translation. Purified and unpurified thioredoxin-gelonin was tested for enzymatic activity in two *in vitro* rabbit reticulocyte lysate assays: the protein synthesis inhibition assay and the RIP-specific N-glycosidase assay. Thioredoxin-gelonin did not exhibit any enzymatic activity in either assay, even at concentrations that were as much as 5000 times higher than was observed using an enzymatically-active isoform of this gelonin as a control.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

A_{260}	absorbance at 260 nm
APS	ammonium persulfate
bp	base pair
BSA	bovine serum albumin
CDR	complementarity determining region
cpm	counts per minute
dNTPs	deoxy-nucleotide triphosphates
DNAse	deoxyribonuclease
DTT	dithiothreitol
E. coli	*Escherichia coli*
HIV	human immunodeficiency virus
IC_{50}	concentration of RIP at which protein synthesis becomes inhibited by 50%
IMAC	immobilized metal affinity chromatography
IPTG	isopropyl-β-$_D$-thiogalactoside
LD_{50}	concentration of RIP necessary to kill 50% of the population
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pI	isoelectric point
PMSF	phenylmethyl sulfonyl fluoride
PPO	2,5-diphenyl-oxazole
psi	pounds per square inch
rEK	recombinant enterokinase (enteropeptidase)
RIPs	ribosome-inactivating proteins
RNAse	ribonuclease
RTA	ricin A-chain
SDS	sodium dodecyl sulfate
S/R	Sarcin/Ricin loop
TBE	Tris-Boric acid-EDTA buffer
TE	Tris-EDTA buffer
TEMED	tetramethylethyldiamine
TLCK	Nα-p-tosyl-$_L$-lysine chloromethyl ketone
TPCK	N-tosyl-$_L$-phenylalanine chloromethyl ketone
Tris	tris(hydroxymethyl)aminomethane
trxA	gene for thioredoxin
UV	ultraviolet

INTRODUCTION

General Background

Ribosome-inactivating proteins (RIPs), such as gelonin, belong to a large and quickly growing family of cytotoxic proteins synthesized from a wide variety of plants and fungi. Once internalized into mammalian cells, RIPs inactivate the ribosomes so that they can no longer carry out protein synthesis. This leads to cell death. Because of their ability to kill cells, many researchers are interested in targeting these molecules toward specific cells (i.e., cancer cells or HIV-infected cells) by conjugating them to antibodies or antibody fragments (referred to as immunotoxins), or ligands (referred to as ligand-toxins).

History of RIPs - Medicinal and Criminal Background

The usage of RIPs for medicinal purposes dates back to ancient times. The Chinese used a preparation (Tian Hua Fen) extracted from the root tuber of the plant, *Trichosanthes kirilowii*, to induce abortion and more recently for treating trophoblastic tumors. The protein responsible for these effects has been found to be the RIP, trichosanthin (Yeung *et al.*, 1988).

The potential of conjugating RIPs to carrier molecules that are capable of delivering them to specific cells has only recently become an area of extensive research. A list of all the uses of these immunotoxins or ligand-toxins for medicinal purposes is too large to be compiled here. However, a few of the successes in clinical trials are as follows: to remove T-cells from bone marrow prior to bone marrow transplants in graft-versus-host disease and in the treatment of colorectal tumors (Stirpe *et al.*, 1992).

In addition to the considerable interest in RIPs for medicinal purposes, RIPs have also become a topic of interest because of their criminal use and potential use as biochemical warfare agents. In 1978, a Bulgarian playwright and Soviet political critic, Georgi Markov, was assassinated in London. The cause of death was a very small, platinum-coated pellet containing the plant RIP, ricin, that had been injected into his leg by his assassin (thought to be a Bulgarian secret agent). The assassin inserted the pellet into the tip of a modified umbrella and injected the pellet into Markov's leg. The event went apparently unnoticed by Markov at the time. Four days later, he was dead (Apple, 1982).

During the Gulf War, Iraq reportedly had 78 pounds of ricin in its biochemical warfare arsenal that may have been intended for use against our military forces (source not disclosed). It has been hypothesized that one molecule of ricin, once internalized into a cell, has the ability to kill that entire cell (Munishkin *et al* 1995). In studies on toxicity to mice, ricin has been estimated to have an LD_{50} (the dosage necessary to kill 50% of the mice) of 3 μg/kg of body mass (Stirpe *et al* 1992). For humans, this would probably be lower. However, assuming that the LD_{50} is the same for humans, 78 pounds would be enough to theoretically kill over 75 million people, each with an average weight of 170 pounds (provided that delivery was 100% effective). Therefore, the development of inhibitors and/or vaccines to protect soldiers and foreign dignitaries is an important national security concern. So for both medicinal purposes (the use of RIPs conjugated to other molecules that target them toward cancer and HIV-infected cells) and for national security purposes (development of inhibitors and/or vaccines), RIPs have been studied extensively over the past two or three decades.

General Information About RIPs

Plant RIPs, such as gelonin, ricin, and trichosanthin, are N-glycosidases, which

hydrolyze the N-glycosidic bond between a specific adenine base and its ribose moiety, thus "depurinating" mammalian rRNA. Figure 1a shows the N-glycosidic bond of adenine. This depurination in the rRNA occurs at a highly conserved loop region, referred to as the Sarcin/Ricin (S/R) loop (Wool *et al.*, 1992). Figure 1b is a diagram based on nuclear magnetic resonance data (NMR) of a 29-nucleotide RNA sequence containing the 17-nucleotide S/R loop and stem structures. This same stem/loop structure is believed to exist in mammalian ribosomes at the site where the RIPs exhibit their activity (Szewczak *et al.*, 1993). Whereas plant RIPs are N-glycosidases which depurinate the rRNA, fungal RIPs, such as α-sarcin and mitogillin, are ribonucleases (RNases), which cleave a specific phosphodiester bond in the rRNA one ribonucleotide away from the adenine described above. Figure 1b depicts the α-sarcin cleavage site in the S/R loop. Either modification (specific depurination by the plant RIPs or specific cleavage of the rRNA backbone by the fungal RIPs) has the same overall effect: the inactivation of ribosomes.

In addition to their ability to inactivate mammalian ribosomes, some RIPs have been cited as having other functions. For example, *Pokeweed* antiviral protein (PAP) is able to inactivate plant rRNA, including its own (Bonness *et al.*, 1994) and *Mirabilis* antiviral protein (MAP) has been shown to inactivate prokaryotic 23 S rRNA (Habuka *et al*,. 1991). The site of depurination in plant rRNA is also a specific adenine base in a region similar in sequence and secondary structure to the S/R loop (Hartley *et al.*, 1991). However, with prokaryotic rRNA, the effects of plant RIPs are only exhibited at much higher concentrations than those reported with eukaryotic ribosomes (Stirpe *et al.*, 1992). Other plant RIPs (PAP-R, saporin 6, and trichokirin) have been cited as having the ability to depurinate mammalian rRNA at multiple sites in intact ribosomes (Barbieri *et al.*, 1992). Lastly, the fungal RIP, α-sarcin, and the plant RIP, trichosanthin, have been cited as having weak deoxyribonuclease (DNase) activity *in vitro*, but only at much higher RIP concentrations as compared with their RNAse and N-glycosidase activities, respectively (Fong *et al.*, 1991). The

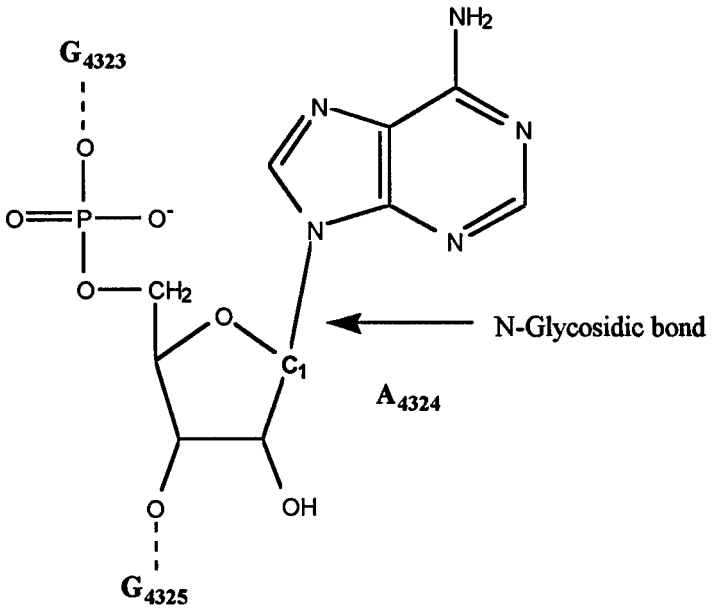
Figure 1

Site of Depurination by RIPs

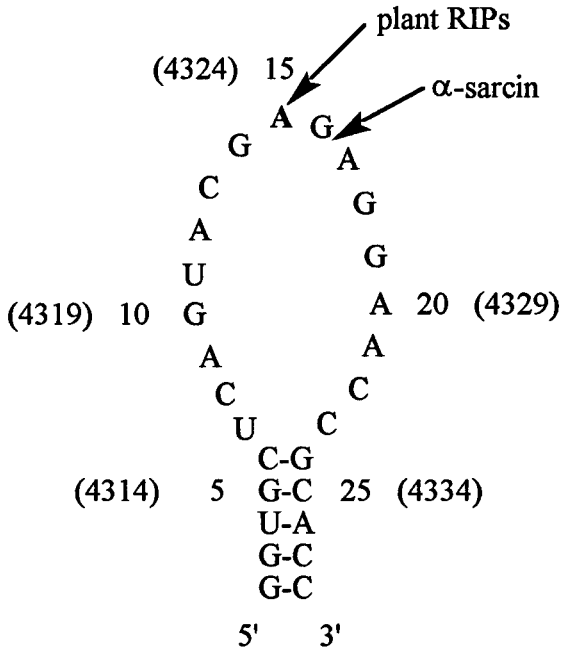
A. The chemical structure of the ribonucleotide, adenosine, is depicted as A_{4324} of the rat liver cytoplasmic 28 S rRNA. The N-glycosidic bond between the adenine base and the ribose sugar is the actual site where the covalent bond is broken by the plant RIPs. The adenine base is subsequently removed from the rRNA by the RIP, thus leaving an aldehyde radical at the C_1 of the A_{4324} ribose (the C_1 is labeled but the aldehyde radical is not shown). Once the 28 S rRNA becomes depurinated, the phosphodiester bonds (shown as dashed lines) between A_{4324} and its two neighboring guanosines become sensitive to aniline at an acidic pH (Endo *et al.*, 1987). Aniline treatment cleaves the 28 S rRNA into two RNA fragments that can be visualized on 3.5% - 4.5% polyacrylamide gels, as described under Methods.

B. A diagram of a 29-oligomer RNA containing the 17-oligonucleotide Sarcin/Ricin (S/R) Loop, as re-drawn from Szewczak *et al.*, 1993. The ribonucleotides are numbered according to their position in the 29-oligomer RNA. In parentheses, the ribonucleotides are labeled with their corresponding position in the rat liver cytoplasmic 28 S rRNA. Plant RIPs depurinate this 29-oligomer at A_{15} (A_{4324} in the 28 S rRNA), whereas α-sarcin cleaves the phosphodiester bond between G_{16} and A_{17} in this 29-oligomer (G_{4325} and A_{4326} in the 28 S rRNA).

A.


G4323
NH2
N
N
N
N
O
O=P—O-
O—CH2
O
C1
N-Glycosidic bond
A4324
O
OH
G4325

B.


plant RIPs
α-sarcin
(4324) 15
A G
G A
C G
A
U G
(4319) 10 G A 20 (4329)
A A
C C
U C
C-G
(4314) 5 G-C 25 (4334)
U-A
G-C
G-C
5' 3'

physiological significance of the DNAse activity seems obscure. Since the focus of this thesis was on plant RIPs, specifically gelonin, only the specific inhibitory effects that plant RIPs have on protein synthesis will be discussed further.

Effects of RIPs on Protein Synthesis

Protein synthesis is a complicated process in which mRNA is translated into polypeptides by macromolecular particles known as ribosomes. There are over 100 components that come together in a precise manner to synthesize polypeptides from mRNA. To better illustrate this complexity and to point out the specific site of action of RIPs, the size and composition of the rat liver cytoplasmic ribosome is described.

The mass of the rat liver cytoplasmic ribosome is approximately 4 x 10^6 Da; it consists of a 40 S subunit (33 different polypeptides and an 18 S rRNA) and a 60 S subunit (49 different polypeptides, 5 S rRNA, 5.8 S rRNA and a 28 S rRNA), according to Voet *et al.*, 1995. The 28 S rRNA of the 60 S subunit contains the S/R loop that is the target of RIPs. The base that becomes hydrolyzed by plant RIPs, $Adenine_{4324}$ (A_{4324}), is thought to lie extended outward in the S/R loop based on NMR spectroscopy of the 29-nucleotide RNA previously mentioned (Szewczak *et al.*, 1993). Out of the 7000 ribonucleotides that are part of each mammalian ribosome (Wool *et al.*,1992), depurination at this single site in the 28 S rRNA renders the entire ribosome inactive.

Protein synthesis generally consists of 3 stages: initiation, elongation, and termination. Although RIPs have been cited as causing a six-fold decrease in the ability of the 40 S and 60 S ribosomal subunits to associate during the initiation stage (Fong *et al.*, 1991), the region of the ribosome that is the target of the RIPs (the S/R loop) is very important in the elongation stage of protein synthesis (Wool *et al.*, 1992). The general consensus in the literature is that depurination of A_{4324} by the plant RIPs inhibits the translocation process during elongation by preventing the GTP-dependent

binding of elongation factor-2 (EF-2) to the ribosomes and subsequent GTP hydrolysis (Fong *et al.*, 1991 and Wool *et al.*, 1992). Without these events occurring, protein synthesis is inhibited at the elongation stage and polypeptides cannot be synthesized. This inhibition subsequently leads to cell death by an unknown mechanism, probably via apoptosis.

Classification and Properties of RIPs

RIPs have generally been classified as Type I or Type II (Stirpe *et al.*, 1992), depending on whether they exist as single polypeptide chains or two dissimilar polypeptide chains connected by a disulfide bond, respectively. An extended classification system has been proposed (Citores *et al.*, 1992) based on the isolation of RIPs with 4 polypeptide chains.

Gelonin, trichosanthin, and PAP are only a few examples of Type I RIPs; most RIPs that have been characterized so far fall into this category. Type I RIPs usually have molecular weights between 26-31 kDa, and exhibit many structural similarities to each other, even though the similarities in their primary amino acid sequences are usually low (typically less than 40%). Type I RIPs typically display the following characteristics: they are glycosylated (on specific asparagine residues), highly stable, strongly basic (isoelectric points or pI often greater than pH 10), and they are potent inhibitors of protein synthesis *in vitro* but have relatively low cytotoxicity *in vivo* since they lack the lectin domain of the Type II RIPs.

Ricin, abrin and modeccin are a few examples of Type II RIPs. These proteins have molecular weights of approximately 60 kDa, and consist of A (active) and B (binding) polypeptide chains connected by a disulfide bond. The two chains are approximately the same size in ricin, and the crystal structures of both chains have been solved by members of Dr. Robertus' laboratory group here at the University of Texas (Rutenber *et al.*, 1991 and Katzin *et al.*, 1991). The A chain is structurally and

functionally similar to that of Type I RIPs, but the A chain can be acidic or basic (pI ranging from pH 4.8 to pH 8.0). The B chain is a lectin that binds to cell surface receptors and facilitates entry of the A chain into the cell. Therefore, these RIPs are highly cytotoxic *in vivo*. The A chains can also be targeted toward specific cells through immunoconjugation or conjugation to ligands, as has been done with the Type I RIPs (Stirpe *et al.*, 1992).

A motif analysis of the amino acid sequences from a variety of RIPs (obtained from the Protein Databank) was performed using the software program, Gene Runner. Motifs are consensus amino acid sequences in a number of proteins that may indicate similar function or three dimensional structure, such as nucleotide binding sites, phosphorylation sites, etc. The five most common motifs found in these RIPs are shown in Table 1. The most interesting finding from this analysis is that gelonin is the only RIP analyzed that does not contain the Shiga/Ricin active site signature motif based on its primary amino acid sequence.

Gelonin

Gelonin is a Type I plant RIP isolated from the seeds of the Asian plant, *Gelonium multiflorum*. This native gelonin has a reported IC_{50} (the concentration of RIP at which protein synthesis becomes inhibited by 50%) of 0.4 nM in the rabbit reticulocyte lysate assay (Stirpe *et al.*, 1992). According to the analysis of the cDNA sequence, gelonin is synthesized as an inactive precursor with either 42 or 46 amino acids on the N-terminus and 19 amino acids on the C-terminus (Nolan *et al.*, 1993). These two extensions are not detected upon isolation of native gelonin from the plant. The N-terminal extension may be necessary for targeting the RIP to specific compartments within plant cells. The C-terminal extension may act as an inactivator of the RIP, as with ricin. This extension allows ricin to be synthesized in an inactive form so that the cells' own ribosomes do not become inactivated. The extension

Table 1

Potential Motifs in RIPs

Gene Runner's motif analysis is based on a comparison of the primary amino acid sequence of each protein with a list of motifs contained in a search table containing the PROSITE database, which is a collection of over 900 protein motifs or signatures. The presence of a motif, as determined by Gene Runner, such as an N-glycosylation site, does not necessarily indicate that the site is actually N-glycosylated in the native protein. The tertiary structures of the RIPs would exclude many of these potential sites from being modified by the enzymes discussed below. For the Shiga/Ricin motif, the numbers in parentheses represent location of this motif within the RIP's primary sequence. For most of the RIPs listed, the Shiga/Ricin motif is between amino acids 150 and 190.

Another motif common to most of the RIPs is the N-glycosylation site. It consists of the consensus pattern (Asn- X- Ser or Thr), in which the Asn becomes glycosylated and X represents any amino acid. Several RIPs, such as gelonin, are glycoproteins (Stirpe *et al.*, 1992), which have been glycosylated in such a manner (at least at some of the potential sites). Other motifs found during this analysis which may or may not be functionally relevant are casein kinase II and protein kinase C phosphorylation sites. These motifs are substrate sites for the respective enzymes, which phosphorylate specific Ser or Thr residues within these motifs. The consensus patterns are as follows: for casein kinase II (Ser or Thr-X-Glu or Asp) and for protein kinase C (Ser or Thr-X-Arg or Lys). The protein kinase C family of enzymes shuttle between the cytosolic face of the plasma membrane and the cytosol; they have been cited as being involved in signal transduction pathways, such as the phosphatidylinositol pathway (Lodish *et al.*, 1995). However, no reference in the literature could be found showing that any isolated plant RIPs were regulated by phosphorylation. In fact, in one study (Sperti *et al.*, 1991), it was shown that although gelonin requires ATP and a high molecular weight component to be present for it to be active (suggestive of a kinase), gelonin itself is not regulated by phosphorylation.

The last common motif, the N-myristoylation site, involves the addition of the C14-saturated fatty acid, myristate, to the first glycine residue in the consensus pattern via an amide linkage. The enzyme responsible for this myristoylation is N-myristoyl transferase. Addition of myristate has been shown to localize proteins to the cytosolic face of the plasma membrane (Lodish *et al.*, 1995). The enzymes responsible for glycosylation, phosphorylation, and myristoylation are only present in eukaryotic organisms.

RIPs / Sites	Shiga/Ricin	N-Glycosylation	N-Myristoylation	Protein Kin C	Casein Kin II
abrin	1 (159-175)	3	7	13	11
agglutinin	1 (1195-1211)	14	35	13	33
B-luffin	1 (176-192)	3	1		3
dianthin	1 (195-211)	1	2	2	9
gelonin-XOMA		2	3	2	2
gelonin-MDA		2	3	2	2
karasurin	1 (155-171)		3	2	3
momorcharin	1 (1175-1191)	8	25	9	28
PAP-A	1 (175-191)	4	1	6	4
PAP-C	1 (1105-1121)	5	23	9	25
PAP-S	1 (170-186)	3	3	6	5
ricin A	1 (172-188)	2	2		3
ricin D	1 (160-176)	1	3	2	6
saporin	1 (171-187)		2	3	6
trichoanguina	1 (153-169)	1	3	3	2
trichosanthin	1 (178-194)		3	2	3
tritin	1 (166-182)		4	3	6

would be removed at a later stage in the secretory pathway (i.e., in the endoplasmic reticulum or Golgi) after the RIP has been translocated out of the cytoplasm where the ribosomes are located (Stirpe *et al.*, 1992 and Nolan *et al.*, 1993).

There are two different gelonin sequences from *Gelonium multiflorum* reported in the literature. A comparison of the two amino acid sequences is shown in Figure 2. The first reported full-length sequence will be referred to here as gelonin-XOMA because it was reported by research scientists at the XOMA Corporation in California (Nolan *et al.*, 1993). The protein contains 251 amino acids, has a molecular weight of 28.2 kDa, and has a pI of pH 9.1, as calculated using Gene Runner. The amino acid sequence was inferred from the cDNA sequence, obtained after isolation and reverse transcription of gelonin mRNA from *Gelonium multiflorum* seeds. The cDNA was fused to the pectate lyase (pelB) leader sequence (a secretory signal sequence in some bacteria), which was under the control of the *Salmonella typhimurium araB* promoter in a pING plasmid. *E. coli* cells were then transformed with this plasmid and induced by addition of $_{L}$-arabinose. The recombinant gelonin-XOMA is reportedly expressed and recovered very efficiently from the medium. It inhibits protein synthesis *in vitro* as efficiently as the native protein isolated from the seeds. Aliquots of the purified gelonin-XOMA were given to us by the MDA research group for use as a positive control, as will be discussed in detail later.

The second gelonin sequence, referred to here as gelonin-MDA, was determined by Dr. Rosenblum's group at M.D. Anderson Cancer Research Center in Houston, Texas, and consists of 258 amino acids (Rosenblum *et al.*, 1995). The protein has a molecular weight of 28.8 kDa and a pI of pH 8.9, as calculated using Gene Runner. The native protein was isolated from *Gelonium multiflorum* seeds and the protein was digested separately by various proteases to produce overlapping polypeptide fragments. The polypeptide fragments were sequenced by Edman degradation and the amino acid sequence of gelonin-MDA was determined. In order to maximize the ability of *E. coli* to synthesize this protein *in vivo*, a 774 base pair (bp)

Figure 2

Amino Acid Sequence Comparison of Gelonin-MDA and Gelonin-XOMA

```
MDA     MGLDTVSFSTKGATYITYVNFLNELRVKLKPEGNSHGIPLLRK
XOMA    MGLDTVSFSTKGATYITYVNFLNELRVKLKPEGNSHGIPLLRK

MDA     G--DDPGKCFVLVALSNDNGQLAEIAIDVTSVYVVGYQVRNRS
XOMA    KCDDPGKCFVLVALSNDNGQLAEIAIDVTSVYVVGYQVRNRS
          |_____|

MDA     YFFKDAPDAAYEGLFKNTIKNPLLFGGKTRLHFGGSYPSLEGE
XOMA    YFFKDAPDAAYEGLFKNTIK---------- TRLHFGGSYPSLEGE

MDA     KAYRETTDLGIEPLRIGIKKLDENAIDNYKPTEIASSLLVVIQMV
XOMA    KAYRETTDLGIEPLRIGIKKLDENAIDNYKPTEIASSLLVVIQMV

MDA     SEAARFTFIENQIRNNFQQRIRPANNTISLENKWGKLSFQIRTSG
XOMA    SEAARFTFIENQIRNNFQQRIRPANNTISLENKWGKLSFQIRTSG

MDA     ANGMFSEAVELERANGKKYYVTAVDQVKPKIALLKFVDKDPE
XOMA    ANGMFSEAVELERANGKKYYVTAVDQVKPKIALLKFVDKDPK
```

Shown in bold print are the differences between the amino acid sequences for the two gelonin molecules, as cited in the literature (Rosenblum *et al.*, 1995 and Nolan *et al.*, 1993). Notice that there is one Cys in the gelonin-MDA and two in the gelonin-XOMA. The two Cys form a disulfide bond (shown as two vertical lines and one horizontal line), as determined from the X-ray structure of native gelonin isolated from *Gelonium multiflorum* (Hosur *et al.*, 1995). The gelonin-MDA has 8 amino acids between Lys_{103} and Thr_{112}, which do not exist in gelonin-XOMA between Lys_{104} and Thr_{105}. Finally, the last amino acid differs between the two molecules.

synthetic gene was engineered, which optimized the number of "high frequency" bacterial codons and increased the number of internal restriction enzyme sites for site-directed mutagenesis studies. This synthetic gelonin gene was inserted into pET-21a and later pET-22b plasmids and transformed into *E. coli*. Dr. Rosenblum kindly provided both of these vectors for use in this research.

Comparison of the amino acid sequences of the gelonin-XOMA and gelonin-MDA sequences in Figure 2 shows two important discrepancies. First, there is one additional lysine and one additional cysteine residue (Lys_{43} and Cys_{44}) in the gelonin-XOMA sequence, which are not found in the gelonin-MDA sequence. Cys_{44} and Cys_{50} form an intrachain disulfide bond; these are the only two cysteine residues in the gelonin-XOMA sequence. Since the reported gelonin-MDA sequence only contains one cysteine (Cys_{49}), there are no disulfide bonds in gelonin-MDA. Secondly, there is a stretch of 8 amino acids (NPLLFGGK) between Lys_{103} and Thr_{112} in the gelonin-MDA sequence, which is not present in the gelonin-XOMA sequence between Lys_{104} and Thr_{105}.

The X-ray structure of native gelonin isolated from *Gelonium multiflorum* has recently been determined. According to the X-ray structure, gelonin was assigned to the $\alpha + \beta$ class of proteins because its N-terminal region (the first 100 amino acids) has predominantly β secondary structure, while the remainder of the protein has predominantly helical structure (Hosur *et al.*, 1995). The amino acid sequence for the native gelonin was identical to the reported sequence of gelonin-XOMA. The crystal structure data has not been submitted to the Protein Databank, so no illustration could be given here. The 8 amino acid peptide sequence reportedly found in the gelonin-MDA during Edman degredation was not present between Lys_{104} and Thr_{105} of the native gelonin crystal structure. Also, the intrachain disulfide bond between Cys_{44} and Cys_{50} that exists in the gelonin-XOMA sequence was clearly discernable from the X-ray data.

Thioredoxin

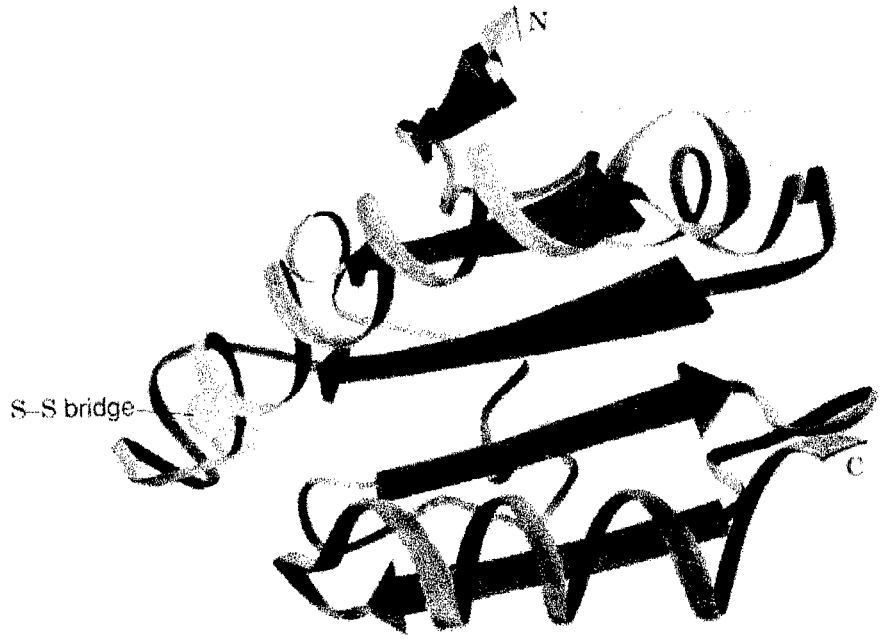
It is highly desirable for the biotechnology industry and for research purposes to produce proteins in *E. coli* (LaVallie *et al.*, 1993) due to the low costs associated with their growth and the high yields of protein that can be obtained. Therefore, it would be a great advantage to be able to synthesize gelonin-MDA in *E. coli*, as has been done with other RIPs, such as ricin A-chain (May *et al.*, 1989) and abrin A-chain (Hung *et al.*, 1994).

The gelonin-MDA gene has been overexpressed under many different conditions in *E. coli*, but the majority of this protein forms insoluble aggregates, known as "inclusion bodies" and exhibits no activity *in vitro* under the assay conditions used in this research. Inclusion bodies consist of improperly folded proteins and often RNA, that aggregate together during protein synthesis (Mitraki *et al.*, 1989). Not much is known about the formation of inclusion bodies and why some eukaryotic proteins can be overexpressed efficiently in bacteria and others cannot. Moreover, some eukaryotic proteins are synthesized in soluble form under certain conditions in *E. coli*, such as incubation at a certain temperature, whereas at other temperatures the same proteins form inclusion bodies (LaVallie *et al.*, 1993). The soluble proteins may or may not exhibit enzymatic activity, but clearly have a higher potential of being active than if the protein formed inclusion bodies. One way to possibly prevent the gelonin-MDA from forming inclusion bodies in *E. coli* was to genetically link the gelonin gene to a second gene which is already known to be expressed in soluble form in these bacteria, such as glutathione-S-transferase, the maltose binding protein, or thioredoxin.

Thioredoxin was chosen for use in this research because it had the following unique properties that made it the most suitable choice. Figure 3 is a diagram of *E. coli* thioredoxin. It is a small, highly stable, thermo-resistant protein. When overexpressed from plasmid vectors, thioredoxin has been shown to accumulate up to

Figure 3

Diagram of Thioredoxin



A diagram of thioredoxin's tertiary structure from Voet *et al.*, 2nd Edition, 1995, Figure 26-14. β–sheets are depicted as arrows and α-helices are depicted as coils. The N and C termini, which are both available for conjugation to other molecules, are labeled. Also shown is the protruding active site loop containing the disulfide bond between Cys_{33} and Cys_{36} (depicted as yellow balls). This protruding loop would be the site of insertion of the peptide aptamers, as described in the introduction.

40% of the total cellular protein and still remain soluble in *E. coli*. Thioredoxin, when fused to many other eukaryotic proteins that were insoluble by themselves when overexpressed in *E. coli*, conferred stability and solubility onto the fused proteins, even at elevated temperatures (LaVallie *et al.*, 1993).

Additionally, thioredoxin's active site loop protrudes outward from the protein. In the pET-32a plasmid, the thioredoxin gene (*trxA*) contains an RsrII restriction enzyme cleavage site, which is located within the coding sequence of the active site loop (at Cys_{33} and Gly_{34}). When short, random oligonucleotides were inserted into the coding sequence at this RsrII cleavage site, the *E. coli*-overexpressed proteins (thioredoxin with random 14 - 25 oligopeptides protruding outward from the active site loop) were highly stable and soluble, whereas the short polypeptides expressed by themselves (without being fused to thioredoxin's active site loop) were insoluble or quickly degraded in *E. coli*. These thioredoxin fusion proteins have been used for screening many types of protein-protein interactions (LaVallie *et al.*, 1993), and have recently been used for expression of a combinatorial library of 20-residue oligopeptides which mimick the complementarity-determining regions (CDRs) of immunoglobulins in a yeast two-hybrid system (Colas *et al.*, 1996).

Aim of this Research

A highly specific, thermostabile ligand-toxin could theoretically be synthesized consisting of two domains separated by a polypeptide linker. The ligand domain would consist of N-terminal thioredoxin with a short polypeptide sequence spliced into to its active site loop, which would mimick the CDR of an antibody and target the molecule toward specific cell populations. The C-terminus of thioredoxin would be genetically fused to a linker polypeptide containing a specific cleavage site(s). The C-terminal domain (consisting of a RIP) would then be genetically fused to the linker region. Once internalized, the fusion protein (or the RIP by itself if it becomes cleaved

at the linker region intracellularly) could catalytically inactivate the ribosomes and lead to cell death.

The effectiveness of such a ligand-toxin would depend on many factors, such as cell surface binding, internalization, translocation to the ribosomes, and of course, its stability and activity once inside the targeted cell. This research was designed to determine if the fusion protein would be soluble and enzymatically active *in vitro*. Specifically, the two questions asked in this research were: "Will fusion to thioredoxin increase the solubility of its fusion partner, gelonin-MDA, when synthesized in *E. coli*?" and "Will the resulting thioredoxin-gelonin fusion protein exhibit specific N-glycosidase activity under the *in vitro* assay conditions used in this research?" In order to answer these questions, the following was accomplished in this research:

1. The gelonin-MDA gene was inserted into the pET-32a plasmid in frame with *trxA*, which is under the control of the T7 promoter. This insertion yielded a vector which codes for a 46 kDa thioredoxin-gelonin fusion protein. This protein has a linker region between thioredoxin and gelonin, which contains both thrombin and enterokinase cleavage sites, as well as a His-Tag for rapid purification. The basic design of this plasmid, referred to as pET-32a/gel, is shown in Figure 4. The amino acid sequence of thioredoxin-gelonin is shown in Figure 5.

2. Thioredoxin-gelonin was overexpressed in *E. coli* BL-21(DE3)pLysS and purified by immobilized metal affinity chromatography (IMAC).

3. Thioredoxin-gelonin was synthesized by *in vitro* coupled transcription/ translation.

4. Thioredoxin-gelonin was cleaved with recombinant enterokinase (rEK) to yield a 29 kDa gelonin and the 17 kDa thioredoxin-linker.

5. The 46 kDa thioredoxin-gelonin and the rEK-cleaved 29 kDa gelonin were tested for enzymatic activity using two *in vitro* assays.

Figure 4

Design of pET-32a/gel Plasmid

Some of the key features of the pET-32a/gel plasmid are shown. The plasmid is 6642 base pairs. The gelonin-MDA gene is shown inserted at the EcoRV site on its 5' terminus and at the HindIII site on its 3' terminus in the pET-32a plasmid (in the same orientation and in frame with the thioredoxin gene, *trxA*). The abbreviations outside the circle represent restriction enzymes followed by their cleavage sites in parentheses. The abbreviations inside the circle and boxed areas represent key sites within the plasmid (*bla* is the gene coding for β-lactamase, ori is the DNA sequence where replication of the plasmid begins, and *lacI* is the gene coding for the *lacUV5* repressor).

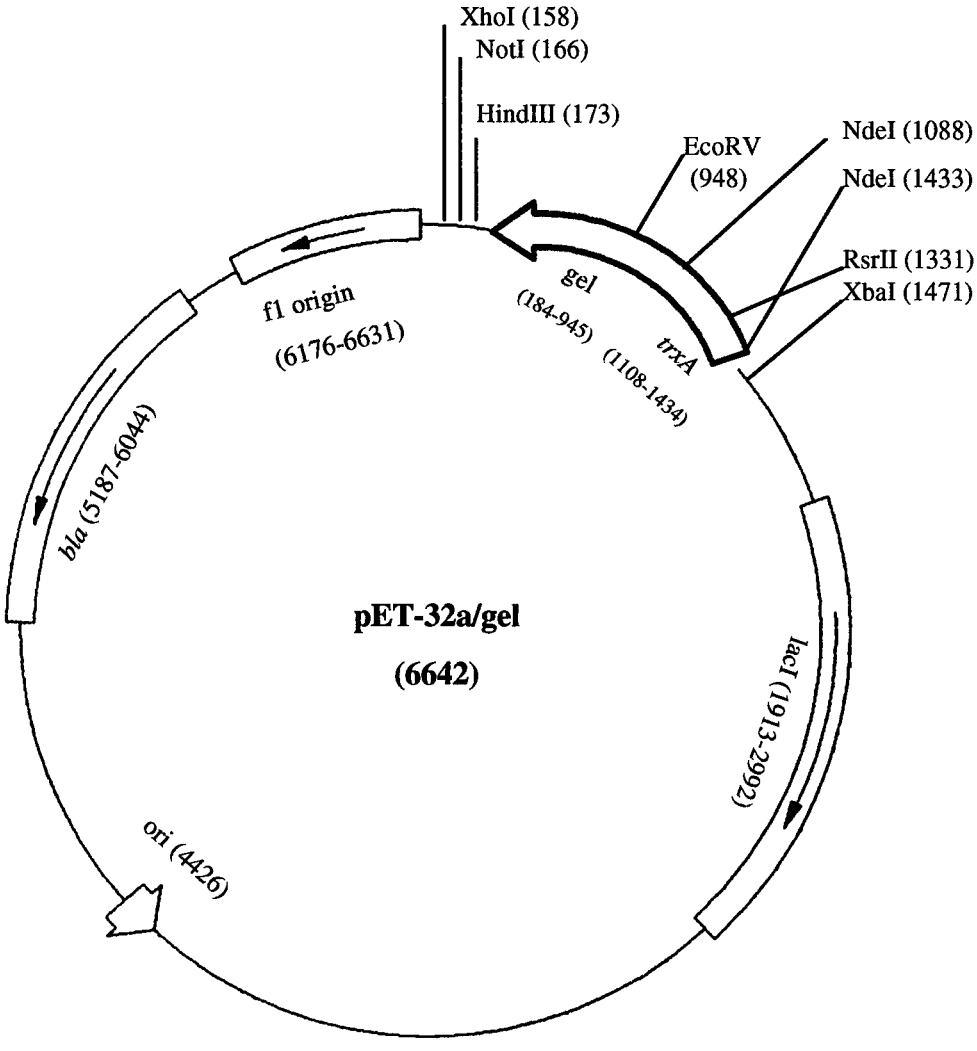

XhoI (158)
NotI (166)
HindIII (173)
EcoRV
(948)
NdeI (1088)
NdeI (1433)
RsrII (1331)
XbaI (1471)
gel
(184-945)
trxA
(1108-1434)
f1 origin
(6176-6631)
bla (5187-6044)
pET-32a/gel
(6642)
lacI (1913-2992)
ori (4426)

Figure 5

Amino Acid Sequence of Thioredoxin-Gelonin

A.

Thioredoxin - 109 aa

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLNIDQ
NPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLA

Linker - 54 aa ↓

GSGSG**HMHHHHHH**SSG**LVPRGS**GM**KETAAAKFERQHMDS**PDLGT**DDDDK**AMADI

His-Tag Thrombin S-Tag Enterokinase

Gelonin-MDA (Val_5 - end) - 254 aa

VSFSTKGATYITYVNFLNELRVKLKPEGNSHGIPLLRKGDDPGKCFVLVALSNDNGQLAEIAID
VTSVYVVGYQVRNRSYFFKDAPDAAYEGLFKNTIKNPLLFGGKTRLHFGGSYPSLEGEKAYRE
TTDLGIEPLRIGIKKLDENAIDNYKPTEIASSLLVVIQMVSEAARFTFIENQIRNNFQQRIRPANN
TISLENKWGKLSFQIRTSGANGMFSEAVELERANGKKYYVTAVDQVKPKIALLKFVDKDPE

B.

Thioredoxin-Gelonin Fusion Protein - 417 aa

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLNIDQ
NPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLAGSGSG**HMHHHHHH**SS
G**LVPRGS**GM**KETAAAKFERQHMDS**PDLGT**DDDDK**AMADIVSFSTKGATYITYVNFLNELR
VKLKPEGNSHGIPLLRKGDDPGKCFVLVALSNDNGQLAEIAIDVTSVYVVGYQVRNRSYFFKD
APDAAYEGLFKNTIKNPLLFGGKTRLHFGGSYPSLEGEKAYRETTDLGIEPLRIGIKKLDENAID
NYKPTEIASSLLVVIQMVSEAARFTFIENQIRNNFQQRIRPANNTISLENKWGKLSFQIRTSGAN
GMFSEAVELERANGKKYYVTAVDQVKPKIALLKFVDKDPE

The thioredoxin-gelonin fusion protein consists of 417 amino acids (aa), has a molecular weight of 46 kDa, and has a calculated pI of pH 6.6. In bold and underlined are the following: the initial methionine of the fusion protein, the His-Tag peptide sequence, the thrombin cleavage site, the S-Tag peptide sequence, and the enterokinase cleavage site (↓). The protein is broken up into the 3 major regions for simplicity (A), and then the entire sequence is shown (B).

Materials and Methods

Materials

The gelonin-MDA gene inserted into pET-21a and pET-22b plasmids and the gelonin-XOMA protein were kindly provided by Dr. Rosenblum (M.D. Anderson Cancer Research Institute, Houston, TX). *E. coli* strain DH-5α was purchased from BRL/GIBCO Life Technologies (Gaithersburg, MD). *E. coli* strain BL-21(DE3)pLysS, the pET-32a plasmid, the His-Bind resin used in Immobilized Metal Affinity Chromatography (IMAC), and the recombinant enterokinase (rEK) were purchased from Novagen (Madison, WI). The S30 fraction prepared from *E. coli* K12 (A19) for *in vitro* coupled transcription/translation was the kind gift of Dr. W. Kudlicki (Dr. Hardesty's laboratory). The Wizard Maxi-Prep Kit for plasmid preparations, the enzymes and buffers for restriction digests, and the rabbit reticulocyte lysate for the N-glycosidase assay were purchased from Promega (Madison, WI). Customer-synthesized oligonucleotide primers were purchased from BioSynthesis Laboratories (Lewisville, TX). Polymerase Chain Reaction (PCR) reagents, including *Thermus aquaticus* (*Taq*) DNA polymerase, were purchased from Fisher Scientific (Pittsburgh, PA). The Fast-Link DNA Ligation and Screening Kit was purchased from Epicentre Technologies (Madison, WI). The nucleotide triphosphates and tRNA for *in vitro* coupled transcription/translation were purchased from Boehringer-Mannheim (Indianapolis, IN). Bacto Tryptone and Bacto Agar were purchased from DIFCO Laboratories (Detroit, MI). Select Yeast Extract, Luria Broth base (LB), ampicillin, lysozyme, imidazole, agarose, nickel sulfate ($NiSO_4$), N,N,N',N'-tetramethylethyldiamine (TEMED), Nα-p-tosyl-$_L$- lysine chloromethyl ketone (TLCK), N-tosyl-$_L$-phenylalanine chloromethyl ketone (TPCK), phenylmethyl sulfonyl fluoride (PMSF), pepstatin A, ethidium bromide, Coomassie Blue R, formamide, 2,5-diphenyl-oxazole (PPO) and all other biochemicals used for the *in*

vitro coupled transcription/translation were purchased from Sigma (St. Louis, MO). Stains-all dye was purchased from Aldrich Chemical Company (Milwaukee, WI). The Untreated Rabbit Reticulocyte Lysate IVT Kit for the protein synthesis inhibition assay and the isopropyl-β-$_D$-thiogalactoside (IPTG) were purchased from Ambion (Austin, TX). Acrylamide, bisacrylamide, bromophenol blue dye, and GBX developer and fixer for autoradiography were purchased from Eastman, Kodak (Rochester, NY). Gel blot paper, GB002, was obtained from Schleicher and Schuell (Keene, NH). Aniline was purchased from JT Baker (Phillipsburg, NJ). DNA molecular weight markers (BstEII Digest of Lambda DNA) were purchased from United States Biochemical Corporation (Cleveland, OH). [^{14}C]-leucine was purchased from ICN Pharmaceuticals (Irvine, CA).

Methods

Molecular Cloning of pET-32a/gel

The pET-22b/gel primer (5'-CATGCTAGATATCGTTAGCT-3'), the T7 terminator primer (5'-GCTAGTTATTGCTCAGCGG-3'), and the pET-22b/gel plasmid were used to amplify the gelonin-MDA gene by PCR. The pET-22b/gel primer was designed for site-directed mutagenesis to create an EcoRV site at the 5' end of the resulting PCR product. See Figure 6. One nucleotide in the pET-22b/gel primer was mutagenized (C→T) to create this EcoRV site (5'-GATATC-3'). The PCR product contained the gelonin-MDA gene beginning at the fifth codon (Val_5).

The PCR product was digested by the restriction enzymes, EcoRV and HindIII, as was the pET-32a plasmid. The gelonin-MDA gene was inserted into the pET-32a plasmid in frame with *trxA*, which is part of the pET-32a plasmid. The resulting pET-32a/gel plasmid contains the gene to synthesize a thioredoxin-gelonin fusion protein, as shown in Figures 4 and 5.

Plasmid Preparation

In order to obtain large amounts of highly purified pET-32a plasmid, the techniques cited in Sambrook *et al.*, 1989, and the Promega Wizard Maxi-Prep Technical Bulletin #139 were combined. In this procedure, *E. coli* BL-21(DE3)pLysS or DH-5α cells containing the plasmid of interest were transferred to 5 ml LB containing ampicillin (60 μg/ml) and incubated in a 37 °C shaker for approximately 8 hours. This culture was then transferred to 2 L of LB containing ampicillin (60 μg/ml) and incubated overnight in a 37 °C shaker. The following morning, the cells were harvested by centrifugation at 6500 rpm for 15 min at 4 °C in a J-21 Beckman Centrifuge using the JA-14 rotor. The supernatant was discarded and the cells were resuspended in 50 ml of 25 mM Tris-HCl, pH 7.8, 10 mM EDTA, and 15% (w/v) sucrose. The bacterial cell walls were digested by adding 100 mg of lysozyme and slowly swirling in an ice bath for 20 min. Cells were lysed by adding 50 ml of 0.2 M NaOH and 1% (w/v) SDS for 10 min on ice. The solution was neutralized by adding 25 ml of 3 M sodium acetate, pH 4.8, and incubating on ice for 10 min. This viscous solution was centrifuged at 17,000 rpm for 20 min at 4 °C in a Sorvall Centrifuge using the SS-34 rotor. The supernatant was transferred into two 150-ml Corex glass containers, and 2.5 volumes of cold, 95% ethanol were added to each. The containers were stored at -20 °C for two hours. The two tubes were centrifuged at 5000 rpm for 30 min at 4°C in a J-21 Beckman Centrifuge using the JA-14 rotor. The supernatant was discarded and the pellets were resuspended in 14.5 ml of TE buffer (10 mM Tris, pH 7.4 and 1 mM EDTA) using a glass pipette. The solution was transferred to a 30-ml Corex tube containing 6 ml of 8.4 M ammonium acetate and incubated on ice for 10 min. The solution was then centrifuged at 10,000 rpm for 20 min at 4°C in a Sorvall Centrifuge using the SS-34 rotor. The pellet was discarded and the supernatant was filtered through Whatman Filter Paper #1 into a cylinder. The filtrate

was then divided equally into two 30-ml Corex tubes. Three volumes of cold, 95% ethanol were added and the tubes were stored at -20 °C overnight. The two 30-ml Corex tubes were then centrifuged at 10,000 rpm for 30 min at 4 °C in a Sorvall centrifuge using the SS-34 rotor. The supernatant was discarded and the pellets were resuspended and combined using 2 - 3 ml of sterile water. At this point, the Promega Wizard Maxi-Prep Protocol (Technical Bulletin #139, Step IV. A. 1 thru 10) was followed as described.

Plasmid Analysis - Plasmid preparations were analyzed by two methods, both of which are discussed in Sambrook *et al.*, 1989 The first was by measuring $A_{260/280}$ in a spectrophotometer at an appropriate dilution. The A_{260} reading was multiplied by the dilution factor and then divided by 20 to yield the concentration of DNA in mg/ml. This method did not provide any information about the purity of each plasmid preparation. The second method provided a qualitative estimate of how much RNA contamination was present in each preparation by using agarose gel electrophoresis to separate the DNA from the RNA.

Agarose Gel Electrophoresis

Agarose gels were prepared in a final volume of 25 ml containing 1X TBE (100 mM Tris base, 100 mM boric acid, 2.5 mM EDTA, pH 8.0), ethidium bromide (1 μg/ml), and the appropriate amount of agarose (i.e., 250 mg for a 1% gel or 300 mg for a 1.2% gel). Aliquots of the plasmid preparation were diluted with autoclaved water, and a 6X gel loading solution (50% (w/v) sucrose, 0.25% (w/v) bromophenol blue) was added to give a final 1X concentration. Plasmid DNA samples were then loaded into the 1% agarose gel and electrophoresed for 25 min at 45 mA in 1X TBE. PCR products and inserts were loaded into a 1.2% agarose gel and electrophoresed for 20 min at 50 - 55 mA in 1X TBE. 5 - 10 μl of DNA molecular weight markers, a BstEII Digest of Lambda DNA (105.8 ng/μl), were electrophoresed in a separate lane

of all agarose gels so that the size of the DNA products and the amount of DNA could be estimated (Sambrook *et al.*, 1989), as described below.

Ethidium bromide intercalates between base pairs and allows the DNA to be visualized and photographed under UV light. The more DNA present, the brighter the bands. The amount of linearized DNA, such as PCR products, inserts and restriction enzyme-digested plasmids, could be determined by comparing the intensity of the DNA band with the intensity of a similar-sized molecular weight marker. UV photographs were taken with a Polaroid camera using Polaroid #52 film.

Polymerase Chain Reaction (PCR)

For small-scale analytical PCR and PCR-screening of transformed colonies, 20 - 25 µl total volume reactions were prepared. Reactions were enlarged to 100 µl for large-scale PCR. All PCR reactions contained 0.25 mM deoxyribonucleotide triphosphates (dNTPs), 10 mM Tris-HCl, pH 8.3 at 25 °C, 50 mM KCl, and 1.5 mM $MgCl_2$. For each 100-µl reaction mixture, 5 units of *Taq* DNA polymerase, 200-400 ng of pET-22b/gel plasmid (template DNA), and 80 - 100 pmol of each primer (pET-22b/gel primer and the T7 terminator primer) were used. The PCR reactions were overlaid with one or two drops of mineral oil. The standard thermalcycler file was used. This consisted of one 2-min cycle at 94 °C, then 29 cycles as follows: 1 min at 94 °C, 2 min at 55 °C, and 3 min at 72 °C (except for the last phase of the final cycle in which the 3 min was extended to 10 min at 72 °C). Aliquots of the PCR reactions were analyzed by 1.2% agarose gel electrophoresis.

Subcloning of Gelonin-MDA Insert into pET-32a

Recovery of PCR Product - The PCR product was recovered by pipetting the aqueous phase of each PCR reaction into a sterile 1.5 ml Eppendorf tube. An equal

volume of chloroform:isoamyl alcohol (24:1) was added and the tube was briefly vortexed 3 times, then centrifuged for 30 seconds. The aqueous (upper) phase was transferred into a sterile 1.5 ml Eppendorf tube containing 2.5 volumes of cold, 95% ethanol. The tube was stored overnight at -20 °C. The tube was centrifuged for 40 min at 16,000 rpm at 4 °C in the Sorvall Centrifuge using the SS-34 rotor. The ethanol was decanted and the pellet was washed with 1 ml of 70% ethanol and centrifuged again for 25 min. The ethanol was decanted. The pellet was air-dried and resuspended in 13 µl of sterile, distilled water.

Restriction Enzyme Digestion - The pET-32a plasmid and the PCR product were digested with the restriction enzymes listed below. For each 20-µl reaction , the reaction mixture contained 2 µg of bovine serum albumin, 600 ng of insert DNA or 1.5 µg of pET-32a plasmid, 20 units each of HindIII and EcoRV, and 2 µl of Promega's 10X Multicore Buffer. Reactions were incubated for 90 min at 37 °C. Entire reaction mixtures were then electrophoresed on 1% agarose gels (for plasmid DNA) or 1.2% agarose gels (for insert DNA).

Recovery of DNA from Agarose Gels - The appropriate bands corresponding to the restriction enzyme-digested DNA (the pET-32a plasmid and the gelonin-MDA insert) were excised from the agarose gels using a surgical scalpel. The plasmid and insert bands were placed in separate 0.5 ml Eppendorf tubes containing autoclaved gel blot paper slurry, prepared as described below. The technique for the recovery of DNA from the agarose gel bands was modified from Chuang *et al.*, 1994.

The slurry was prepared by cutting a 2 cm x 4 cm piece of gel blot paper #GB004 (Schleicher and Schuell #31530), into 5 mm x 5 mm squares, which were then placed in a 50 ml screw-cap tube containing 30 ml of 10 mM Tris-HCl, pH 8.0, 0.5 M NaCl, and 1 mM EDTA. After allowing the slurry to sit for 10 min, the tube was shaken vigorously until the gel blot paper was uniformly expanded. One hole was poked through the bottom of several 0.5 ml Eppendorf tubes using a 23-gauge needle. The slurry was added to each tube and packed down to a height of 3 - 4 mm. The

caps were then closed and the tubes were placed in a glass jar and autoclaved for 20 min. The tubes were stored in the cold room.

The agarose gel bands containing plasmid or insert DNA were cut into small squares and placed on top of the autoclaved gel blot paper in separate 0.5 ml Eppendorf tubes. These tubes were placed inside sterile, 1.5 ml Eppendorf tubes (minus the cap) and centrifuged at 12,000 X g for 10 min at 4 °C. The DNA passes through the slurry and collects into the 1.5 ml Eppendorf tube (along with water, TBE and ethidium bromide that was contained in the gel matrix). The 0.5 ml Eppendorf tube containing the remaining agarose and gel blot paper was discarded. 2 M KCl was added to a final concentration of 100 mM. The DNA was precipitated by adding 2.5 volumes of 95% ethanol and storing the tubes overnight at -20 °C. The 1.5 ml tubes were then centrifuged for 40 min at 16,000 rpm at 4 °C in a Sorvall Centrifuge using the SS-34 rotor with 1.5-ml adapters. The ethanol was discarded. Each pellet was dried completely and then resuspended in the appropriate amount of sterile water to be used in the ligation reactions.

Ligation of Insert into Plasmid - The Fast-Link DNA Ligation and Screening Kit protocol was followed as closely as possible. The protocol required a 2:1 molar ratio of insert:plasmid DNA if both termini were cohesive, and a 10:1 molar ratio if both ends were blunt. The gelonin-MDA insert and the cut pET-32a plasmid each had one blunt end (EcoRV) and one cohesive end (HindIII). So an arbitrary 3:1 molar ratio of insert:plasmid DNA was used. Total volume of the ligation reaction mixture was 15 µl, which contained 150 ng of cut pET-32a, 59 ng of cut gelonin-MDA insert, 1.0 µl of T4 DNA ligase, 1.5 mM ATP, and 1.5 µl of Epicentre's 10X Ligation Buffer. The reaction was carried out for 1 hour at room temperature.

Preparation of Competent Cells - *E. coli* BL-21(DE3)pLysS and DH-5α were separately made competent for electroporation according to the procedure cited in Sambrook *et al.*, 1989, with minor modifications. A 500-ml flask containing 50 ml of autoclaved S.O.B minus Mg^{+2} (2% (w/v) Bacto Tryptone, 0.5% (w/v) Select Yeast

Extract, 2.5 mM KCl, and 10 mM NaCl) was inoculated with the selected *E. coli* cells containing no plasmids. The 50-ml culture was shaken overnight at 37 °C with vigorous aeration. The following morning, 2 ml of the overnight culture was transferred to 500 ml of the S.O.B broth described above. The cells were incubated for 2 - 3 hours until A_{550} was approximately 0.8. The cells were harvested by centrifugation at 6500 rpm for 10 min at 4 °C in a J-21 Beckman Centrifuge using the JA-14 rotor. The cells were resuspended in 400 ml of ice cold, sterile 10% (v/v) glycerol and centrifuged at 6500 rpm for 20 min as above. The supernatant was immediately decanted and the pellet was resuspended in 2.5 ml of sterile 10% (v/v) glycerol. Cells were aliquoted (100 and 200 µl) into sterile, 0.5 ml Eppendorf tubes and frozen in liquid nitrogen. The aliquots were then stored at -70 °C until needed for transformation.

Transformation by Electroporation - The transformation reaction mixture contained 5 µl of the ligation reaction and 20 µl of competent cells. The cells were transformed using a BRL/GIBCO Life Technologies' Cell-Porator *E. coli* Electroporation System in accordance with the operators manual. The cells were then pipetted into a sterile 1.5 ml Eppendorf tube containing 500 µl of sterile LB (no antibiotics) and incubated in the 37 °C shaker bath for one hour. Then an appropriate amount of this cell culture was transferred to an agar plate containing LB and ampicillin (60 µg/ml). Cells were spread out over the agar using a sterilized glass rod. The agar plates were incubated at room temperature for 20 min so that the cells would adhere to the agar surface. The plates were then inverted and placed overnight in the 37 °C incubation chamber.

PCR-Screening of Transformed Colonies - The following morning, colonies were replica-plated and PCR-screened to determine which colonies had PCR products that were the predicted size. For the pET-32a/gel plasmid, the PCR product using the T7 promoter and T7 terminator primers was calculated to be 1454 bp. For the PCR-screening, each colony was collected onto the tip of a sterile toothpick and gently

touched to the surface of a second agar plate containing LB and ampicillin (60 μg/ml). This second plate or "replica plate" was marked on the back with horizontal and vertical lines, thus forming grid squares. Each grid square was designated by a letter of the alphabet. The toothpick, which still contained the majority of the colony, was transferred to a sterile, 0.5 ml Eppendorf tube containing 40 - 50 μl of sterile water and labeled according to the corresponding letter on the replica plate. The colony was transferred from the toothpick into the water. All tubes were placed in a boiling water bath for 5 min so that the plasmid DNA would be released as the *E. coli* cells lysed. PCR reaction mixtures were set up so that the final volumes were 20 μl; half this volume consisted of each plasmid DNA solution. All other reactants and the standard thermal cycler file utilized were previously described in the section on PCR. The PCR products were analyzed on a 1.2% agarose gel and a UV photograph was taken.

In Vivo Overexpression of RIPs (Analytical)

Select *E. coli* BL-21(DE3)pLysS colonies that contained the correct-sized PCR product were grown to mid-log phase and induced. The cells were harvested and lysed, and the lysates were analyzed by 10% SDS-PAGE to determine if the overexpressed protein was the calculated size for the gelonin from pET-21a/gel (29 kDa) or thioredoxin-gelonin from pET-32a/gel (46 kDa). One tube containing 5 ml of LB with ampicillin (60 μg/ml) was innoculated and grown overnight at 37 °C. The following morning A_{600} was measured at 1:10 dilution. The overnight culture was diluted to A_{600} = 0.1 into two tubes containing 5 ml LB with ampicillin (60 μg/ml). The cells were incubated for about 2 hours until A_{600} was between 0.5 - 0.8. At this point, 100 mM IPTG was added to a final concentration of 0.5 mM to one 5-ml culture, but not the other. The induced cell culture was initially incubated for 1 ½ hours at 37 °C. In subsequent studies, induced cell cultures were incubated under varying conditions: duration of induction, incubation temperature, IPTG

concentration. One-ml aliquots from induced and control cultures were pipetted into 1.5 ml Eppendorf tubes and the cells were harvested by centrifugation for 10 min at 8000 X g at 4 °C. The medium was decanted. For experiments analyzing the solubility of the overexpressed proteins, cells were resuspended in resuspension buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, and 1 mM PMSF) and then subjected to freeze-thaw and sonication. Freeze-thaw was carried out by freezing the cells at -70 °C and then slowly thawing them on ice. Sonication was carried out using three 10-second bursts; each burst was followed by incubation on ice for one min. For experiments analyzing the amount of overexpression, cells were resuspended in 50 - 200 µl of 2X Sample Buffer (125 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 2% (w/v) SDS, 0.08% (w/v) bromophenol blue dye, and 0.2 M dithiothreitol-DTT), depending on the concentration of cells. Appropriate amounts of each sample were boiled for 5 min to lyse the cells and then analyzed by 10% SDS-PAGE.

10% SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

All protein samples, whether purified or in cell lysates, were analyzed by 10% SDS-PAGE. The 10% resolving gel was prepared by combining 3.1 ml of 30:0.8% (w/v) acrylamide:bisacrylamide, 2.63 ml of 1.5 M Tris-HCl, pH 8.8, 94 µl of 10% (w/v) SDS, and 3.5 ml of distilled water. The above solution was degassed for one hour to eliminate trapped gases, and then 38 µl of 10% (w/v) ammonium persulfate (APS) and 18 µl of TEMED were added. The resolving gel was then poured between two 10 X 10 cm glass plates and allowed to polymerize for one hour. The 4% stacking gel was prepared by adding 381 µl of 30:0.8% (w/v) acrylamide:bisacrylamide, 287 µl of 1.25 M Tris-HCl, pH 6.8, 30 µl of 10% (w/v) SDS, 2.15 ml of distilled water, 16 µl of 10% (w/v) APS, and 14 µl of TEMED.

Aliquots of cell lysate or purified proteins were boiled for 5 min and then loaded into 10% SDS-polyacrylamide gels and electrophoresed at 110 V for the

desired amount of time. The running buffer used for SDS-PAGE consisted of 25 mM Tris, pH 8.3, 190 mM glycine, and 0.5% (w/v) SDS. Gels were then carefully removed from the glass plates and placed into a fixing solution (45% methanol and 10% acetic acid) for 10 min. Gels were stained using the fixing solution containing 0.25% (w/v) Coomassie Brilliant Blue R dye for 25 min, and then destained with the fixing solution for several hours. Gels were dried on a gel dryer.

Analysis of the Solubility of the RIPs Synthesized *In Vivo*

Cell lysates from the *In Vivo* Overexpression of RIPs (Analytical) experiment described above were centrifuged in accordance with Novagen's pET System Manual in order to collect any inclusion bodies. This was accomplished by centrifugation at 12,000 X g for 15 min. The volume of the supernatant (soluble fraction) was measured and pipetted into a separate Eppendorf tube. An aliquot of the supernatant was removed and 2X Sample Buffer was added to the aliquot. The pellet (insoluble fraction) was resuspended using an equal volume of resuspension buffer as supernatant that was removed. An aliquot of this fraction was removed and 2X Sample Buffer was added. Both aliquots were analyzed by 10% SDS-PAGE, as previously described. By comparing the total amount of overexpressed protein in the pellet with the total amount in the supernatant, percentages of gelonin and thioredoxin-gelonin in both fractions were compared.

***In Vivo* Overexpression of RIPs (Large-Scale)**

The procedure was similar to that described above for the analytical method. One test tube containing 5 ml of LB plus ampicillin (60 μg/ml) was inoculated with BL-21(DE3)pLysS containing either the pET-21a/gel or pET-32a/gel plasmid, and incubated for 6 - 8 hours. This culture was transferred to 100 ml of LB with ampicillin

(60 μg/ml) and grown overnight at 37 °C. The following morning, A_{600} was measured at 1:10 dilution. The appropriate volume from the overnight culture was transferred to 2 L of LB containing ampicillin (60 μg/ml) so that the A_{600} was 0.1. The cells were incubated for approximately 2 hours until A_{600} was 0.5 - 0.8. To induce the cells, 100 mM IPTG was added to a final concentration of 0.5 mM, and the culture was incubated for 2 ½ hours at 37 °C. Cells were harvested by centrifugation for 10 min at 8000 rpm in the J-21 Beckman Centrifuge using the JA-14 rotor. If thioredoxin-gelonin was to be purified from the soluble fraction by immobilized metal affinity chromatography (IMAC), the medium was poured off and the cells were resuspended in 10 ml of 1X Binding Buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, and 5 mM imidazole) containing the following protease inhibitors: 1 mM PMSF, 0.1 mM TLCK, 0.2 mM TPCK, and 20 μg/ml pepstatin A. If the soluble fraction was to be tested in the RIP-specific N-glycosidase assay described later, the cells were resuspended in 10 ml of 2X N-glycosidase assay buffer (40 mM Tris-HCl, pH 7.5, 20 mM $Mg(OAc)_2$, 200 mM NH_4Cl, and 2 mM DTT) containing the same concentrations of protease inhibitors. The cells were lysed by passing them through a French Press twice at 800 psi. The lysate was centrifuged at 39,000 X g at 4 °C for 15 min. To purify thioredoxin-gelonin from the *E. coli* soluble fraction, the remainder of the supernatant was subjected to IMAC under non-denaturing conditions, as described below.

The insoluble proteins were resuspended in 1X Binding Buffer containing 6 M urea and incubated for 1 hour at 37 °C (to denature the inclusion bodies). After the 1 hour incubation, the tube was stored on ice for 30 min and then centrifuged at 20,000 X g for 15 min. To purify thioredoxin-gelonin, the supernatant was subjected to IMAC under denaturing conditions, as described later.

Purification of Thioredoxin-Gelonin (Non-Denaturing Conditions)

Since thioredoxin-gelonin contained a His-Tag (an 8 amino acid peptide

containing 7 histidines) in the linker region between the thioredoxin and gelonin domains, the fusion protein could be purified by IMAC using Novagen's His-Bind Resin. The resin was loaded into a column to a settled bed volume of 2.5 ml. The chromatography was carried out as described in Novagen's pET System Manual. The IMAC column was charged and equilibrated using 3 volumes of deionized water, 6 volumes of 1X Charge Buffer (100 mM $NiSO_4$), and 3 volumes of 1X Binding Buffer. After centrifuging the cell lysate from the large-scale overexpression experiment described above, the supernatant was loaded on top of the column bed. The flow through fraction was collected. To separate the *E. coli* proteins from thioredoxin-gelonin, 6 volumes of 1X Binding Buffer and 3 volumes of 1X Wash Buffer (20 mM Tris-HCl, pH 7.9, 60 mM imidazole, 500 mM NaCl) were added sequentially. To elute thioredoxin-gelonin, 3 volumes of 1X Elute Buffer (20 mM Tris-HCl, pH 7.9, 1 M imidazole, 500 mM NaCl) were added and 1-ml fractions were collected. After each change of buffer solution, the column was closed for 10 min to allow for column equilibration. Aliquots from each chromatographic step were analyzed by 10% SDS-PAGE. The eluted fractions containing thioredoxin-gelonin were concentrated using a Centricon tube, molecular weight cutoff of 10 kDa. By diluting and then concentrating with 1X ribosomal buffer (20 mM Tris-HCl, pH 7.5, 10 mM $Mg(OAc)_2$, and 1 mM DTT), aliquots of thioredoxin-gelonin were prepared for the protein synthesis inhibition assay.

Purification of Thioredoxin-Gelonin (Denaturing Conditions)

The IMAC column was set up, charged, and equilibrated in the same manner as described above. The procedure for chromatography under denaturing conditions is described in Novagen's pET System Manual. Denatured inclusion bodies were solubilized as described in the large-scale overexpression experiment. The supernatant was then loaded on top of the column bed. To purify the denatured thioredoxin-

gelonin from other macromolecules present, 10 volumes of 1X denaturing Binding Buffer (20 mM Tris-HCl, pH 7.9, 5 mM imidazole, 500 mM NaCl, and 6 M urea) and 3 volumes of 1X denaturing Wash Buffer (20 mM Tris-HCl, pH 7.9, 24 mM imidazole, 500 mM NaCl, and 6 M urea) were added sequentially. To elute the denatured thioredoxin-gelonin fusion protein, 3 volumes of 1X denaturing Elute Buffer (20 mM Tris-HCl, pH 7.9, 300 mM imidazole, 500 mM NaCl, and 6 M urea) were added and 1-ml fractions were collected. Aliquots from each chromatographic step described were analyzed by 10% SDS-PAGE. The urea, imidazole, and NaCl were gradually removed from the eluted fractions using the same Centricon tubes already described. Aliquots of thioredoxin-gelonin were prepared for the N-glycosidase assay and for recombinant enterokinase cleavage by repeatedly diluting and concentrating with the respective buffers.

In Vitro Coupled Transcription/Translation

The method developed by Zubay and refined by Kudlicki (Kudlicki *et al.*, 1992) for *in vitro* coupled transcription/translation in a static system was used with minor modifications to synthesize the 29 kDa gelonin and 46 kDa thioredoxin-gelonin proteins. T7 RNA polymerase was added to the reaction mixture since the genes for these two proteins are each under the control of the T7 promoter in the pET-21a/gel and pET-32a/gel plasmids, respectively.

S30 fractions prepared from *E. coli* strain K12 (A19) were the kind gift of Dr. W. Kudlicki. The total volume of each reaction mixture was either 30 µl or 60 µl. Each reaction mixture contained 50 mM Tris-acetate, pH 8.2, 10 mM $Mg(OAc)_2$, 36 mM NH_4OAc, 72 mM KOAc, 2 mM DTT, 2% (w/v) polyethylene glycol, 1 mM ATP, 0.8 mM each of GTP, UTP, and CTP, 0.5 mM cAMP, 25 mM phosphoenolpyruvate, 11.7 ng/µl pyruvate kinase, 33.3 ng/µl folinic acid, 83 µM [^{14}C]-leucine, 200 µM of each of the other 19 amino acids, and 0.667 ng/µl of *E. coli* tRNA (Boehringer), 33.3

ng/μl rifampicin, 1.2 - 2.4 A_{260} units of S30 ribosomal fraction, 16.7 ng/μl T7 RNA polymerase, and 1 - 2 μg of uncut plasmid DNA. Negative controls contained all of the components listed above minus the plasmid DNA. Reaction mixtures were incubated for 30 min at 37 °C. Upon completion of the 30 min incubation, 2 - 5 μl aliquots were removed and the procedure for trichloroacetic acid (TCA) Filtration/Precipitation was followed in order to determine the amount of radioactivity, which is proportional to the amount of protein synthesized.

TCA Filtration/Precipitation

The procedure cited in Ambion's Retic Lysate IVT™ manual was followed. Aliquots from the *in vitro* coupled transcription/translation reaction mixture above or from the protein synthesis inhibition assay listed below were pipetted into 10 mm X 75 mm glass test tubes containing 500 μl of water to stop the reaction. Then 500 μl of 1 M NaOH and 1.5% H_2O_2 were added to each test tube in order to deacylate the aminoacylated tRNAs and bleach any colors that could cause quenching during the scintillation counting, respectively. The test tubes were incubated for 5 min in a 37 °C water bath. The tubes were then placed on ice and 1 ml of 25% (w/v) TCA was added to precipitate the proteins. After 10 min on ice, the solutions were filtered through Schleicher & Schuell glass fiber filters #34 using a Millipore Filtration apparatus. The test tubes were rinsed twice with 5% (w/v) TCA and poured over the glass fiber filters. The filters were dried in a 160 °C oven for 10 min and placed into 5 ml of scintillation fluid (0.5% (w/v) 2,5-diphenyl-oxazole in toluene). Radioactivity was measured in a Beckman LS100C Scintillation Counter.

Analysis of the Solubility of the RIPs Synthesized *In Vitro*

Upon completion of the *in vitro* coupled transcription/translation and removal

of the aliquots described above, the remainder of the reaction mixtures were centrifuged at 150,000 X g in a Beckman airfuge to separate the soluble proteins from the insoluble RIPs (full-length, insoluble RIPs and less than full-length, ribosome-bound nascent RIPs). The soluble fractions were removed and the insoluble fractions were resuspended in 10 µl of 2X Sample Buffer. An equal amount of 2X Sample Buffer was added to each 10 µl aliquot from the soluble fractions. Samples were analyzed by 10% SDS-PAGE. Gels were stained, and dried as previously described. The gels were subjected to autoradiography for the desired amount of time and the film was developed using developer, water, and fixer. The bands on the autoradiogram that corresponded to only the full-length RIPs in each fraction (soluble and insoluble) were excised from the gel and dissolved in 200 µl of 30% hydrogen peroxide at 60 °C overnight. Each RIP/hydrogen peroxide mixture was transferred to 5 ml of 5% Biosolve in scintillation solution and the amount of radioactivity was measured in a Beckman LS100C scintillation counter. Percentages of full-length RIPs in the soluble and insoluble fraction were calculated by dividing the amount of radioactivity in either fraction by the total amount of radioactivity in both fractions.

Recombinant Enterokinase (rEK) Cleavage

Thioredoxin-gelonin contains the enterokinase recognition sequence and cleavage site (DDDDK↓) in the linker region near the start of the gelonin sequence. See Figure 5. Aliquots of the eluted fractions were equilibrated as previously described using 1X rEK cleavage buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, and 2 mM $CaCl_2$). 50 - 100 µl reaction mixtures containing 25 - 50 µg of denatured thioredoxin-gelonin and 0.5 - 1.0 units of rEK were incubated at room temperature overnight. Aliquots of the overnight reaction mixtures were analyzed by 10% SDS-PAGE to determine the extent of cleavage. Other aliquots were tested in the N-glycosidase assay, as described later in this section.

Protein Synthesis Inhibition Assay

The procedure used was very similar to the protein synthesis protocol listed in Ambion's Retic Lysate IVT™ manual. However, 20 µl reactions were prepared using 14 µl of Ambion's Untreated Rabbit Reticulocyte Lysate, 1.0 µl of [^{14}C]-leucine (330 Ci/mol), 1.0 µl of Ambion's Master Mix (minus leucine), and various amounts of RIPs. In a separate reaction mixture, the respective buffer (minus RIPs) was added instead of the RIPs. This reaction mixture yielded the data for 100% protein synthesis, since no RIPs were present and all other ion concentrations were the same. All reactions were prepared in 0.5 ml Eppendorf tubes and incubated in a 30 °C water bath for 24 or 30 min. 2 µl aliquots were removed from each reaction mixture at 8 or 10-min intervals. The amount of radioactivity incorporated into reticulocyte proteins was measured using the procedure for TCA Filtration/Precipitation described above.

RIP-Specific N-Glycosidase Assay

The total volume of the reaction mixtures were 20 µl containing final concentrations of 1X N-glycosidase assay buffer (10 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl, 10 mM $Mg(OAc)_2$, and 1 mM DTT), 16 µl of Promega's Untreated Rabbit Reticulocyte Lysate, and varying amounts of the purified RIPs (i.e., gelonin-XOMA or the 46 kDa IMAC-purified thioredoxin-gelonin). For the reactions using non-purified gelonin and thioredoxin-gelonin, 20 - 26 µl reaction mixtures were set up containing final concentrations of 1X N-glycosidase assay buffer, 16 µl of Promega's reticulocyte lysate, and 2.5 - 10 µl of each *E. coli* soluble fraction containing the overexpressed RIPs. All reactions were incubated for 10 min at 30 °C. To stop each reaction and begin the RNA extraction, 3 volumes of 1.5% (w/v) SDS were added.

Phenol Extractions - At room temperature, an equal volume of phenol was added to each reaction mixture above. Each mixture was briefly vortexed several times and then centrifuged for 1 min. The aqueous (upper) phase was pipetted into a sterile 0.5 ml Eppendorf tube without removing any of the precipitated proteins that lie between the phenol:water interface. The phenol phase was washed using 50 µl of autoclaved distilled water to ensure maximum recovery of the RNA. The aqueous phase was again recovered and combined with the first aqueous phase. A second phenol extraction was carried out by adding an equal volume of phenol to the combined aqueous phase. After pipetting the aqueous phase into a sterile 1.5 ml Eppendorf tube, the phenol phase was washed with 80 µl of autoclaved distilled water to ensure maximum recovery of RNA. After brief, repeated vortexing and centrifugation, the aqueous phase was combined with the previous aqueous phase.

Chloroform Extraction - At room temperature, an equal volume of chloroform (about 200 µl) was added to each combined aqueous phase, vortexed, and centrifuged as described above. The aqueous (upper) phase was pipetted into a sterile, 1.5 ml Eppendorf tube and the chloroform phase was washed with 100 µl of autoclaved distilled water, vortexed and centrifuged. The aqueous phases were combined.

First Ethanol Precipitation - 3 M NaOAc, pH 4.8, was added to a final concentration of 300 mM, and then 3 volumes of cold, 95% ethanol were added. The RNA was stored overnight at -20 °C. The solution was centrifuged for 30 min at 14,000 rpm in the Sorvall Centrifuge at 4 °C using the SS-34 rotor with 1.5-ml tube adapters. The ethanol was decanted without disturbing the barely visible RNA pellet, which was then washed with 300 µl of 70% ethanol and centrifuged as described above for 10 min. The ethanol was removed with a sterile syringe, air-dried for 2 min, and then resuspended in 30 µl of sterile, distilled water.

Aniline Cleavage - In order to cleave the sensitive phosphodiester bond at the site of depurination in the 28 S rRNA, 150 µl of aniline solution, pH 4.6 (by volume 9% aniline and 16% glacial acetic acid) was added and incubated for 10 min at 40 °C.

Second Ethanol Precipitation - A second ethanol precipitation was carried out overnight as described for the first ethanol precipitation. The pellet was resuspended in 15 µl of 0.2X TBE containing 8 M Urea.

Preparation of Samples for Electrophoresis - Samples were heated for 5 min at 55 °C. One µl of 0.25% (w/v) bromophenol blue dye was added to each sample. Samples were loaded on 4% or 4.5% polyacrylamide gels and electrophoresed for 30 min at 180 - 200 V.

4% Polyacrylamide Gel Electrophoresis

Each gel was prepared using 1.33 ml of 30:08 (w/v) acrylamide:bisacrylamide solution, 1 ml of 10X TBE, and 7.6 ml of water, 40 µl of 10% (w/v) APS, and 20 µl of TEMED. The gel was poured between two 10 X 8.5 cm mini-gel glass plates. No stacking gel was used.

Gels were pre-run at 180 V for 30 min in 1X TBE. The samples, which were prepared as described above under RIP-specific N-glycosidase assay, were loaded and electrophoresed for 30 min at 180 - 200 V in 1X TBE.

Each gel was carefully transferred from the glass plates and placed into a large Petri dish containing 50 ml of 1X TBE and ethidium bromide (5 µg/ml), then shaken gently for 15 min. The 1X TBE was removed and the gel was photographed while still in the Petri dish under UV light using Kodak #57 film. After taking the UV photographs, gels were placed in 50 ml of Stains-all solution containing 1% (w/v) Stains-all in 60:40 formamide:water and stained by shaking gently for 30 - 45 min in the dark. The gels were destained by removing the 1X Stains-all solution, adding 50 ml of distilled water, and shaking gently in the presence of light until the background dye was removed. The gels were dried on a gel dryer for 30 - 45 min.

Results

Construction of the pET-32a/gel Plasmid

Plasmid Preparations - Large-scale plasmid preparations were repeated at various times throughout this research to obtain the pET-22b/gel plasmid (the source of template DNA for the PCR) and the pET-32a plasmid (the vector for insertion of the gelonin-MDA gene). Both *E. coli* strains, BL-21(DE3)pLysS and DH-5α, containing either of the plasmids listed above were used for these preparations. Approximately 150 - 500 μg of plasmid DNA were obtained from each 2-L preparation. This is not a very high yield of plasmid DNA; yields ranging from 2 - 5 mg of high copy number plasmids from a 500-ml culture are common (Sambrook *et al.*, 1989). A high copy number plasmid, such as pUC and pGEM, indicates that the plasmid exists in many copies in the organism, whereas other plasmids exist in low copy number. The reasons for this disparity are not clear. The relatively low yields obtained suggest that these pET plasmids are low copy number plasmids.

The purest plasmid preparations with the highest yields of plasmid DNA were obtained when the DH-5α cells were used in comparison to the BL-21(DE3)pLysS cells. This is not surprising considering the fact that the BL-21(DE3)pLysS strain is an *endA* positive strain. The *endA* gene codes for the 12 kDa, endonuclease I protein, which is directed into the periplasm of these bacteria. Upon lysis of the BL-21 cells, this endonuclease, which uses double-stranded DNA as its substrate, could be largely responsible for the low yield of plasmid DNA, as discussed in the Wizard Maxiprep Kit technical bulletin.

Polymerase Chain Reaction - The pET-22b/gel primer and the T7 terminator primer were used to amplify the gelonin-MDA gene from the pET-22b/gel plasmid, as shown in Figure 6a. The PCR product, which was calculated to be 890 bp, contained

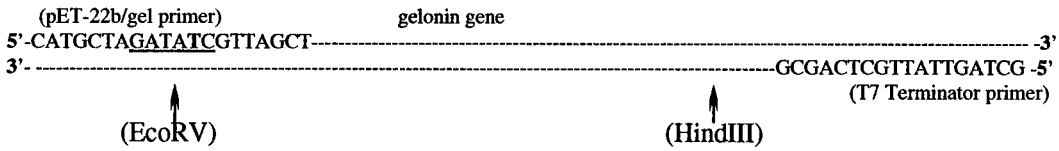
Figure 6

PCR Product from the pET-22b/gel Plasmid

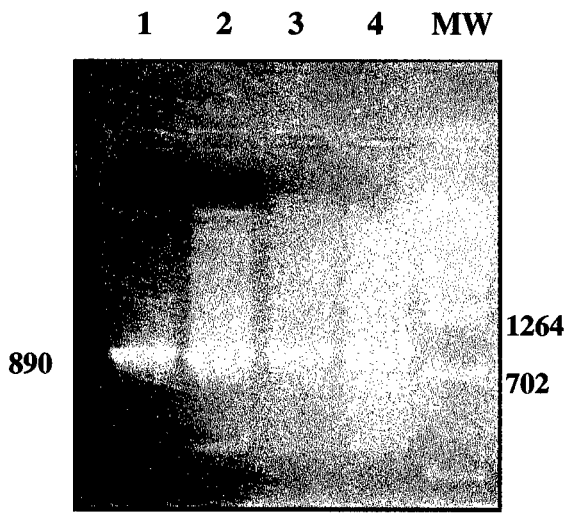
A. Diagram of the expected PCR product using the two primers shown and the pET-22b/gel plasmid as the template DNA. The pET-22b/gel primer was designed for site-directed mutagenesis to generate an EcoRV restriction enzyme site near the 5' end of the 890 bp PCR product. One nucleotide in the pET-22b/gel primer, a C→**T**, was altered as compared to the gene sequence in the pET-22b/gel plasmid in order to create this EcoRV site (5'-GATATC-3').

B. UV photograph of the PCR products after agarose gel electrophoresis. Lanes 1 and 2 contain 4 µl and 8 µl, respectively, from the first PCR reaction mixture. Lanes 3 and 4 contain 4 µl and 8 µl, respectively, from the second PCR reaction mixture. The major PCR product is labeled to the left of the photograph. The last lane contains 8 µl of BstEII-digested Lambda DNA molecular weight markers (105.8 ng/µl). The pertinent molecular weight markers are labeled to the right of the gel. Based on the intensity of the 702 bp molecular weight marker, the amount of 890 bp PCR product in each of the other four lanes was estimated, as listed below the photograph.

A.



B.



Lane	pET-22b/gel PCR product	Estimated DNA	Concentration
1	4 µl	33 ng	[8.3 ng/µl]
2	8 µl	66 ng	[8.3 ng/µl]
3	4 µl	40 ng	[10 ng/µl]
4	8 µl	80 ng	[10 ng/µl]

the gelonin-MDA gene beginning at the fifth codon onward. From two 100-µl reaction mixtures, an estimated 1.8 µg of PCR product was obtained based on comparisons made between the brightness of the PCR product with the brightness of known amounts of the 702 bp molecular weight marker. Figure 6b is a UV photograph of the agarose gel after electrophoresis. The estimated amounts and concentrations of the 890 bp PCR product obtained are listed below the photograph.

Restriction Enzyme Digestion - The pET-32a plasmid and the 890 bp PCR product described above were digested with EcoRV and HindIII. After restriction enzyme digestion, the linearized plasmid DNA was predicted to be 5871 bp and the insert was predicted to be 771 bp. The results for the restriction enzyme digestion of the plasmid DNA are not shown. Figure 7a depicts the PCR product with the positions of the restriction enzyme cleavage sites numbered. Figure 7b shows the UV photograph of the agarose gel taken after restriction enzyme digestion and electrophoresis.

The digestion appeared to have gone very close to completion. However, if only the HindIII enzyme cleaved the 890 bp PCR product, the larger product would be 781 bp. If both EcoRV and HindIII cleaved the PCR product, the larger product would be 771 bp; this was the correct product needed for the ligation. The difference between a 781 bp and 771 bp linear DNA fragment is not readily distinguishable by agarose gel electrophoresis.

There was one intense band in lanes 1 and 2 that migrated about the same distance as the 702 bp DNA marker. This was slightly faster than expected for a 771 bp linear DNA fragment, but there were higher concentrations of ions present in the restriction enzyme-digestion reaction mixtures than were present in the molecular weight marker DNA. This difference in ion concentrations could have increased the electrophoretic mobility of the restriction enzyme-digested DNA (Sambrook *et al.*, 1989). Since the band labeled 771 bp in lanes 1 and 2 was so thick, it made it even

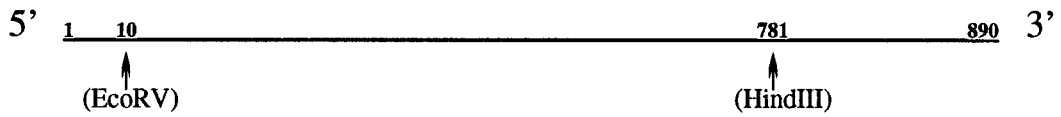
Figure 7

Restriction Enzyme Digestion of the PCR Product

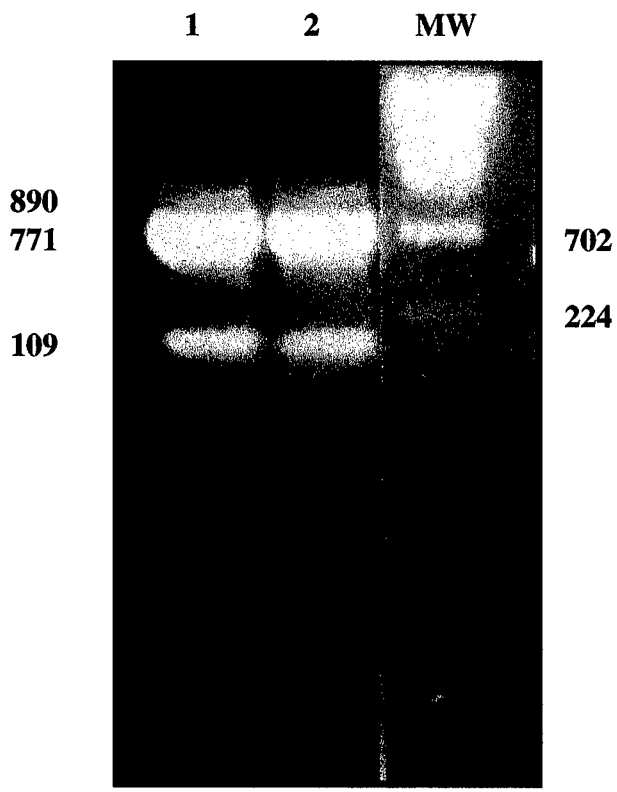
A. Diagram of the coding strand of the PCR product listing the restriction enzyme cleavage sites. EcoRV cleaves (↑) after the 10^{th} bp and HindIII cleaves (↑) after the 781^{st} bp of the 890 bp PCR product. The expected sizes for the fragments generated from complete digestion using these two restriction enzymes are as follows: (771, 109, and 10 bp). The 771 bp product is the insert needed for cloning into pET-32a.

B. UV photograph of the restriction enzyme digestion products after 1.2% agarose gel electrophoresis. In lanes 1 and 2, three bands can be seen and are labeled to the left of the photograph. The major product is labeled 771 bp; these intense bands in lanes 1 and 2 were excised from the gel and used as the gelonin insert for the ligation. The 702 and 224 bp molecular weight markers are labeled to the right of the photograph.

A.



B.



more difficult to distinguish between a 781 bp and a 771 bp product. So the intense band in lanes 1 and 2 were excised from the gel for use in the ligation.

Ligation of Insert into Plasmid and PCR-Screening of Transformed Colonies - Ligation of the gelonin DNA insert into the linearized pET-32a plasmid involved the formation of four new phosphodiester bonds between adjacent 5'-phosphate and 3'-hydroxyl groups (two bonds at the EcoRV cleavage site and two bonds at the HindIII cleavage site). Bacteriophage T4 DNA ligase was used to catalyze the formation of these phosphodiester bonds because of its ability to join blunt-ended (EcoRV) DNA fragments, as well as cohesive (HindIII) DNA fragments under typical reaction conditions (Sambrook *et al.*, 1989). The rate of the ligation of DNA fragments is determined by the concentration of DNA containing compatible termini.

In general, for ligation of DNA fragments with cohesive termini, a 2:1 molar ratio of insert:plasmid is cited as yielding the greatest number of recombinants. For ligation of DNA fragments with blunt-ended termini, a 10:1 molar ratio of insert:plasmid is recommended (Epicentre Technologies' Fast-Link DNA Ligation and Screening Kit protocol) in order to yield the greatest number of recombinants. Lower ratios of insert:plasmid favor the recircularization of the plasmid, and higher ratios of insert:plasmid favor oligomerization of the insert. Since the insert and plasmid DNA fragments used for this ligation each contained one blunt-ended termini and one cohesive termini, an arbitrary 3:1 molar ratio of insert:plasmid DNA was used.

For the 15-µl ligation reaction mixture, 150 ng of the 5871 bp, restriction enzyme-digested pET-32a plasmid were used. Assuming 330 as the average molecular weight per nucleotide, the molecular weight for the 5871 bp plasmid is 3.87 X 10^6. The number of nmol of plasmid DNA in 150 ng was determined by dividing 150 ng by the molecular weight (3.87 X 10^6), which equals 3.88 X 10^{-5} nmol of plasmid. Since a 3:1 molar ratio of insert:plasmid DNA was needed, the molar amount of plasmid DNA (3.88 X 10^{-5} nmol) was multiplied by 3, which equals 1.16 X 10^{-4}

nmol of insert. The molecular weight of the 771 bp insert is 5.1 X 10^5. Multiplying the number of nmol of insert needed by its molecular weight equals 59 ng of insert. So 150 ng of plasmid and 59 ng of insert were used for ligation.

E. coli BL-21(DE3)pLysS cells were transformed by electroporation and then plated overnight on an agar plate containing LB and ampicillin. Fourteen colonies grew on the plate. Only one out of the 14 colonies (7%) contained the correct construct, as described below. The majority of the colonies contained recircularized pET-32a plasmid without any inserts. This indicates that a higher than 3:1 molar ratio of insert:plasmid probably would have yielded more recombinants during the ligation.

This also indicates that the restriction enzyme digestion of the pET-32a plasmid was incomplete. It's unlikely that the adjacent 5'-phosphate and 3'-hydroxyl groups between a blunt-ended and a cohesive termini could become ligated. The more believable explanation is that some of the pET-32a plasmid was digested by one of the two restriction enzymes, but not by both. This would have resulted in a 5900 bp linearized plasmid with two compatible termini, which is not readily distinguishable from a 5871 bp linearized plasmid (the major product from digestion of pET-32a by both EcoRV and HindIII) by agarose gel electrophoresis. During the ligation, these two compatible termini could have been re-joined causing pET-32a to recircularize.

The expected size of the PCR product using the T7 promoter and T7 terminator primers and pET-32a/gel as the source of template DNA is 1454 bp. A diagram of the 1454 bp PCR product is shown in Figure 8a. Figure 8b shows the UV photograph of the agarose gel after electrophoresis of the PCR products from 7 out of the 14 colonies. In lane 3, the PCR product migrated slower than the PCR products in the other six lanes; this PCR product migrated close to the 1374 bp DNA marker. Due to the higher ion concentrations in the PCR reaction mixtures, the PCR products migrated slightly faster in comparison to the DNA molecular weight markers.

Figure 8

PCR Product from the pET-32a/gel Plasmid

A. Diagram of the expected PCR product obtained by using the T7 promoter and T7 terminator primers and the pET-32a/gel plasmid as the template DNA. The PCR product is 1454 bp and contains the features listed below. The top strand is the coding strand. The linker region contains the DNA sequence coding for the His-Tag region, the S-Tag region, and the thrombin and enterokinase cleavage sites. The *trxA* gene contains 109 codons, the linker region contains 54 codons, and the gelonin gene contains 254 codons.

B. UV photograph of the PCR-screening products after agarose gel electrophoresis. The significant molecular weight markers are labeled to the right of the photograph. The 1264 and 1371 molecular weight markers migrated as one single band under the conditions used. In lane 3, the PCR product migrated slower than the PCR products generated by the other 6 colonies (lanes 1, 2, 4, 5, 6, and 7) and migrated close to the expected size for a 1454 bp PCR product. Sequencing of the plasmid DNA from this colony confirmed that the gelonin gene was correctly inserted into the pET-32a plasmid.

A.

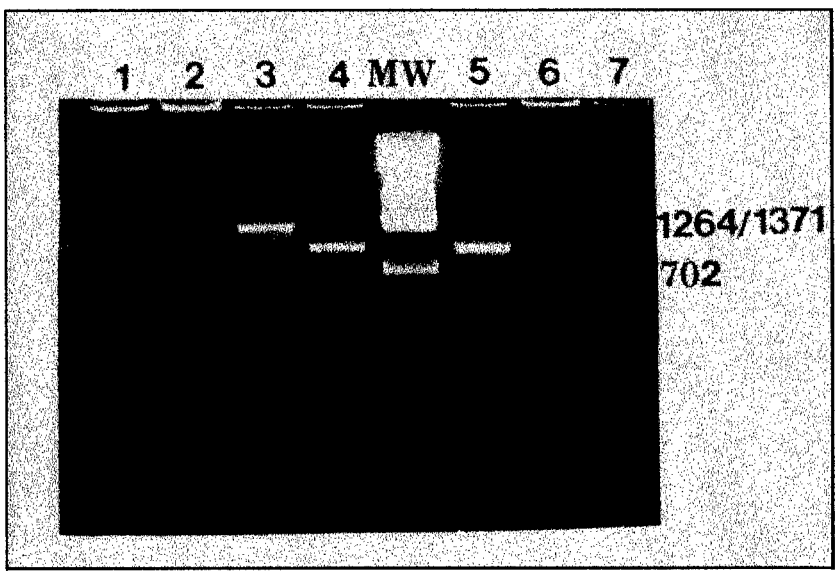
(T7 Promoter primer) → (*trxA*) (linker) (gel)

5'----------3'

3'----------5'

← (T7 Terminator primer)

B.



DNA Sequencing - The colony described above was grown in LB and ampicillin, and a plasmid preparation was done as described under Methods. As a final confirmation that the pET-32a/gel construct was correct, the plasmid was taken to the DNA Sequencing Facility at the University of Texas at Austin. The S-Tag primer (5'-CGAACGCCAGCACATGGACA-3'), which anneals to the linker region between *trxA* and the insert, was used for the DNA sequencing. This allowed part of the linker region and approximately 674 of the 774 bases in the synthetic gelonin gene to be read accurately. These results confirmed that the gelonin-MDA gene was correctly inserted into the pET-32a plasmid in frame with *trxA*. Results from the DNA Sequencing Facility are not shown.

In Vivo and *In Vitro* Expression of the RIPs

E. *coli* strain BL-21(DE3)pLysS and pET vectors - *E. coli* BL-21(DE3)pLysS cells, which are commonly used for protein overexpression, contain the gene for the bacteriophage T7 RNA polymerase in their genome. This gene is under the control of the *lacUV5* promoter, which binds 1,6-allolactose (the inducer of this promoter). The expression of the gene is repressed under normal growth conditions, so little or no T7 RNA polymerase is present. The binding of allolactose transiently activates transcription of the gene for T7 RNA polymerase. IPTG, however, is a non-hydrolyzable analog of allolactose. IPTG also binds to *lacUV5*, but constitutively activates transcription of the gene(s) under the control of this promoter.

The pET vectors contain a DNA sequence, the T7 promoter region, just upstream of the multiple cloning site, which is the site of insertion for the gene of interest. The pET vectors also contain the gene for β-lactamase under a bacterial promoter. This enzyme is secreted into the periplasmic space of *E. coli*, where it has the ability to cleave the antibiotic, ampicillin, which would otherwise be toxic to *E.*

coli. This allows for selection of bacteria that contain the pET plasmid, since only these bacteria will have ampicillin resistance.

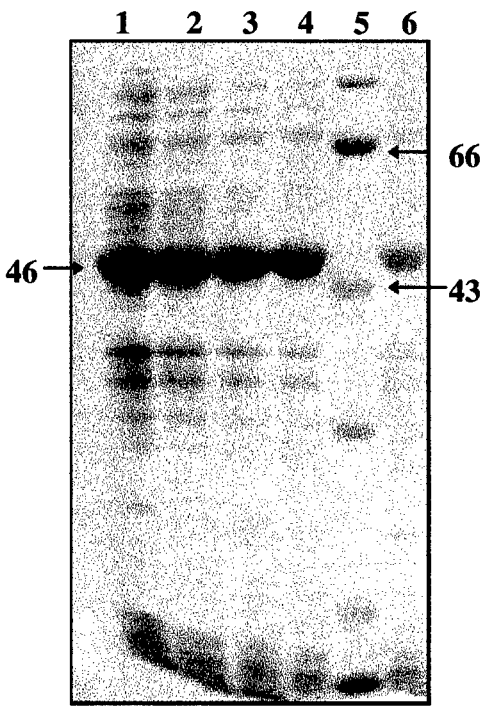
Upon addition of IPTG to logarithmically growing BL-21(DE3)pLysS cells, the T7 RNA polymerase gene is constitutively expressed. This polymerase binds strongly to its specific DNA binding sequence, the T7 promoter, which is only present in the pET plasmids and not in *E. coli* chromosomal DNA. The binding of T7 RNA polymerase to the T7 promoter is followed by transcriptional activation of the gene under its control. Provided that there is a start codon (AUG) that is the proper distance from the ribosome binding site in the mRNA transcript, translation of the mRNA will occur and the protein of interest will be expressed. Since the strength of the affinity between the viral T7 RNA polymerase and the viral T7 promoter results in high levels of transcriptional activation, large amounts of mRNA are usually produced. Consequently, some proteins have been shown to accumulate up to 40% of the total cellular protein in *E. coli* (Novagen's pET System Manual).

In vivo Overexpression of Thioredoxin-Gelonin (Analytical) - First, a small-scale (analytical) experiment was performed. Comparison of the lysates from non-induced and induced cells clearly showed overexpression of a 46 kDa protein from the induced cell lysate when analyzed by 10% SDS-PAGE. Next, a time course experiment was carried out to determine the amount of overexpression over a 4-hour induction period at one hour time intervals. As shown in Figure 9, a steady increase in the amount of overexpressed thioredoxin-gelonin was observed, reaching a maximum at 3 hours. This protein accumulated to approximately 30% of the total cellular protein. In lane 6, the non-induced cell lysate also contained an increased amount of 46 kDa protein, but not as much as the induced cells in lanes 1-4. The reason why this occurred is that even in the absence of IPTG, some T7 RNA polymerase inevitably becomes synthesized, as mentioned in the pET System Manual. It is then able to bind to the T7 promoter sequence in the plasmid and transcribe the gene under

Figure 9

Photograph of the SDS-Polyacrylamide Gel

(Time Course of Induction)



BL-21(DE3)pLysS cells containing the pET-32a/gel plasmid were harvested from 0 to 4 hours after induction and the lysates were analyzed by 10% SDS-PAGE, as shown. (Lane 1) Cells were harvested at 4 hours after induction. (Lane 2) Cells were harvested at 3 hours. (Lane 3) Cells were harvested at 2 hours. (Lane 4) Cells were harvested at 1 hour. (Lane 5) Molecular weight markers are shown (from top to bottom in kDa- 94, 66, 43, 30, 21, 14). (Lane 6) Cells were harvested without being induced. The 66 kDa and 43 kDa markers are labeled on the right, and the overexpressed 46 kDa protein is labeled on the left.

its control. The pET-32a/gel plasmid contains the *lacI* gene under the control of a bacterial promoter. This gene codes for the repressor of the *lacUV5* promoter, which controls the expression of the T7 RNA polymerase gene. In the absence of IPTG, the protein product of *lacI* is supposed to prevent the expression of the T7 RNA polymerase gene, but apparently was not very effective in this case.

In vivo Overexpression of Gelonin (Analytical) - A small-scale (analytical) experiment was performed. When analyzed by 10% SDS-PAGE, the lysate from the induced cells clearly contained a much larger amount of overexpressed 29 kDa protein compared with the lysate from the non-induced cells (results not shown).

In Vitro Coupled Transcription/Translation - The largest yield of *in vitro*-synthesized thioredoxin-gelonin was obtained when 1 μg of pET-32a/gel plasmid was added per 30-μl reaction mixture. Using [^{14}C]-leucine, specific activity 40 Ci/mol (S.A. 40), the amount of radioactivity incorporated into the thioredoxin-gelonin molecules was 130, 840 cpm. Since each molecule contains 38 leucine residues, this equals 43 pmol or 2 μg of protein per 30-μl reaction mixture.

The largest yield of *in vitro*-synthesized gelonin was obtained when 1.2 μg of pET-21a/gel plasmid was added per 30-μl reaction mixture. Using [^{14}C]-leucine (S.A. 160), the total amount of radioactivity was 310, 000 cpm. Since each gelonin molecule contains 24 leucine residues, this equals 40 pmol or 1.15 μg of protein per 30-μl reaction mixture.

Purification of Thioredoxin-Gelonin by IMAC and rEK Cleavage

Purification by IMAC (Non-Denaturing Conditions) - For this type of immobilized metal affinity chromatography (IMAC), the metal (Ni^{+2}) is bound to Novagen's His-Bind resin. Proteins that contain an oligopeptide sequence with 6 - 10 histidine residues will bind strongly to the resin due to the affinity between the

electron-deficient Ni^{+2} and the electron-rich imidazole ring of each histidine. Addition of a buffer containing a high concentration of imidazole, as described under Methods, causes the His-Tag protein to elute from the column.

Only 60 μg of thioredoxin-gelonin were eluted by IMAC (non-denaturing conditions) from an overexpressed 2-L culture harvested at A_{600} = 1.1. This reflects the extremely low percentage of thioredoxin-gelonin in the soluble fraction when overexpressed *in vivo*. This protein was then diluted and concentrated with 1X ribosomal buffer to remove the imidazole and NaCl before the protein could be tested in the protein synthesis inhibition assay. Much of this protein was lost or degraded (probably due to its instability) during the dilution and concentration process. There was not enough protein remaining for the recombinant enterokinase cleavage reaction, as discussed later. Since the majority of thioredoxin-gelonin was insoluble, the insoluble fraction was subjected to IMAC under denaturing conditions.

Purification by IMAC (Denaturing Conditions) - The insoluble fraction was resuspended in 1X denaturing Binding Buffer (1X BB containing 6 M urea), incubated for 60 min, and then centrifuged to separate the soluble proteins from the insoluble proteins, as described under Methods. Analysis by 10% SDS-PAGE indicated that approximately 2/3rd of the thioredoxin-gelonin remained in the soluble fraction after the centrifugation (results not shown). This soluble fraction was subjected to IMAC under denaturing conditions. Approximately 20 mg of purified thioredoxin-gelonin were removed from the column by the 1X denaturing Wash Buffer; and 18 mg of this protein was eluted by the 1X denaturing Elute Buffer. The amount of His-Bind resin used (2.5 ml bed volume) was only enough to bind 20 mg of protein containing a His-Tag under optimum conditions (Novagen's pET System Manual). So recovery of 18 mg of protein in the elute step was about as efficient as could be expected. Had more resin been used, more of this protein could have been recovered in the eluted fractions.

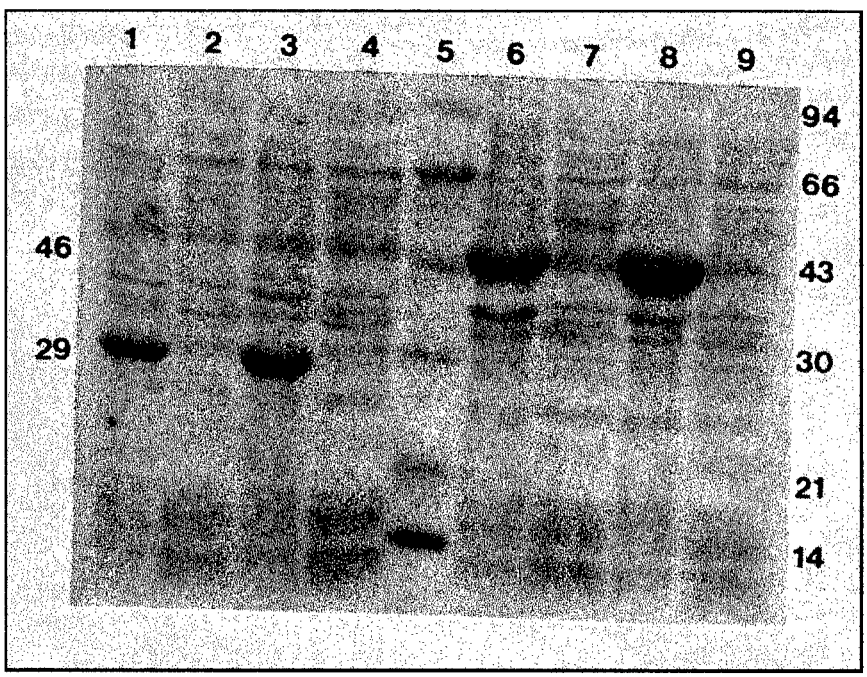
rEK Cleavage of Thioredoxin-Gelonin - After removing the imidazole, NaCl, and urea that was present in the eluted fractions (from IMAC under denaturing conditions), 20 - 40 μg of thioredoxin-gelonin was used as the substrate for rEK cleavage. Analysis of aliquots by 10% SDS-PAGE showed two products, as expected. The larger product was the 29 kDa gelonin and the smaller product was the 17 kDa thioredoxin/linker molecule (results not shown). One important observation was that the band corresponding to the 17 kDa thioredoxin/linker product appeared to contain approximately 4 times the amount of Coomassie dye compared to the band corresponding to the 29 kDa gelonin product. Since a 1:1 ratio would be expected, this could indicate that the gelonin was labile and was somehow degraded during the overnight incubation. Shorter reactions resulted in incomplete cleavage by rEK.

Solubility Experiments

In Vivo Solubility Experiments - Two *E. coli* BL-21(DE3)pLysS colonies containing the pET-21a/gel plasmid and two colonies containing the pET-32a/gel plasmid were grown logarithmically to A_{600} = 0.65 and then induced in order to overexpress gelonin and thioredoxin-gelonin, respectively. Cells were harvested, lysed by sonication, and then centrifuged to separate the insoluble and soluble proteins. Aliquots from each fraction were analyzed by 10% SDS-PAGE. Figure 10 is a photograph of the gel. Almost all of the gelonin (lanes 1 and 3) and almost all of the thioredoxin-gelonin (lanes 6 and 8) are in the insoluble fraction. From these results, there does not appear to be an increase in the amount of soluble thioredoxin-gelonin (lanes 7 and 9) compared to the amount of soluble gelonin (lanes 2 and 4). These results suggest that fusion to thioredoxin did not increase the solubility of gelonin when overexpressed in *E. coli*, at least in the design used in this research (the design of thioredoxin-gelonin with 54 amino acids separating thioredoxin from gelonin).

Figure 10

Photograph of the SDS-Polyacrylamide Gel of the Overexpressed RIPs from *E. coli* (Analysis of Solubility)



Two BL-21(DE3)pLysS colonies containing pET-21a/gel and two colonies containing pET-32a/gel were induced. The cells were harvested, sonicated, and the soluble and insoluble fractions were separated by centrifugation. Lanes 1 and 3 are the insoluble fractions and lanes 2 and 4 are the soluble fractions from the two colonies containing pET-21a/gel. Lanes 6 and 8 are the insoluble fractions and lanes 7 and 9 are the soluble fractions from the two colonies containing pET-32a/gel. Almost all of the 29 kDa gelonin molecules are in the insoluble fractions (lanes 1 and 3), as are almost all of the 46 kDa thioredoxin-gelonin molecules (lanes 6 and 8). Molecular weight markers are in lane 5 and are labeled to the right of the photograph in kDa.

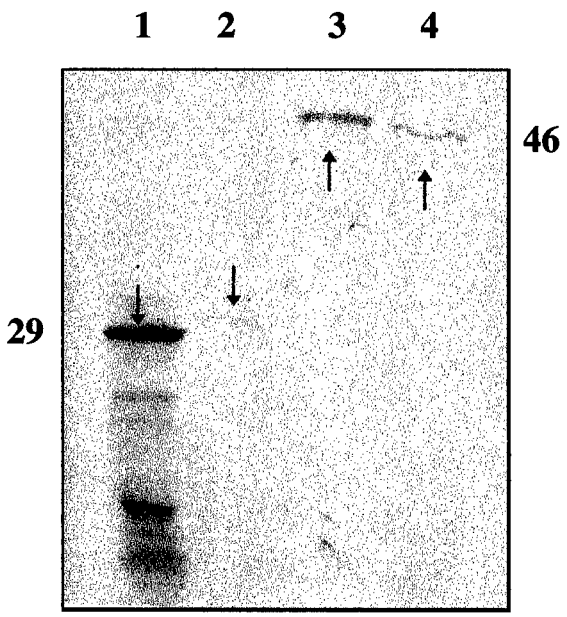
In Vitro Solubility Experiments - Gelonin and thioredoxin-gelonin were synthesized by *in vitro* coupled transcription/translation in an *E.coli*-derived system. The soluble proteins were separated from the insoluble proteins (consisting of full-length insoluble RIPs and less than full-length, ribosome-bound, nascent polypeptides) by centrifugation. All of the resuspended pellet and 10 µl out of the 55 µl soluble fraction were analyzed by 10% SDS-PAGE and subsequent autoradiography. Figure 11 is a photograph of the autoradiogram. The full-length proteins are labeled to the left of the photograph. Clearly, there is much more soluble 46 kDa thioredoxin-gelonin in lane 4 than there is soluble 29 kDa gelonin in lane 2. There is much more insoluble 29 kDa gelonin in lane 1 than there is 46 kDa thioredoxin-gelonin in lane 3. Comparing the amount of insoluble versus soluble gelonin in lanes 1 and 2, respectively, almost all of the gelonin appears to be insoluble. In contrast, there is approximately half as much 46 kDa thioredoxin-gelonin in the soluble fraction versus the insoluble fraction in lanes 3 and 4, respectively. Taking into account that only 10 µl out of 55 µl of each soluble fraction was analyzed, whereas each entire insoluble fraction was analyzed, the increase in the amount of soluble thioredoxin-gelonin versus the amount of soluble gelonin is even more pronounced.

In order to quantitate the amount of protein in each fraction, the bands corresponding to only the full-length RIPs in each fraction were excised from the gel and incubated overnight in hydrogen peroxide. These solutions were transferred to 5 ml of scintillation fluid containing 5% Biosolve and the amount of radioactivity was measured as described under Methods. As shown in Table 2, only 22% of the full-length gelonin was soluble, whereas 71% of the full-length thioredoxin-gelonin was soluble. These results suggest that fusion to thioredoxin did increase the solubility of gelonin when synthesized in this bacteria-derived, *in vitro* coupled transcription/translation system.

Figure 11

Photograph of the Autoradiogram of *In Vitro*-Synthesized RIPs

(Analysis of Solubility)



Lanes 1 and 2 contain the insoluble and soluble fractions, respectivley, for the 29 kDa gelonin (↓) synthesized by *in vitro* coupled transcription/translation. Lanes 3 and 4 contain the insoluble and soluble fractions, respectively, for the 46 kDa thioredoxin-gelonin (↑) synthesized by *in vitro* coupled transcription/translation. The entire insoluble fractions were loaded into lanes 1 and 3, whereas only 10 µl of the 55 µl of each soluble fraction were loaded into lanes 2 and 4. Quantitative measurements of the amount of [^{14}C]-leucine incorporated into each full-length RIP per each fraction are listed in Table 2.

Table 2

Analysis of the Solubility of the *In Vitro*-Synthesized RIPs

RIPs	Fraction	Measured cpm	Total cpm	Per Fraction
Gelonin	Insoluble	42,280 cpm	42,280 cpm	78%
Gelonin	Soluble	2,210 cpm	12,155 cpm	22%
Thioredoxin-gelonin	Insoluble	6,635 cpm	6,635 cpm	29%
Thioredoxin-gelonin	Soluble	3,020 cpm	16,600 cpm	71%

After the incubation period, the *in vitro* coupled transcription/translation reaction mixtures containing the synthesized RIPs were centrifuged to separate the soluble RIPs from the insoluble and ribosome-bound (incomplete, nascent) RIPs. The soluble fractions were removed and 10 µl out of 55 µl were analyzed by 10% SDS-PAGE, while the entire pellet was resuspended and analyzed in the same manner. The gel was subjected to autoradiography and the bands corresponding to the full-length RIPs in each fraction were excised from the gel. The bands were dissolved in hydrogen peroxide overnight. Scintillation fluid containing 5% Biosolve was added to each tube and the amount of radioactivity was measured as described under Methods. The third column in the table shows the measured radioactivity in counts per minute (cpm) for each fraction. In the fourth column, the amount of total soluble protein was calculated by multiplying the measured cpm by 5.5, since only a 10 µl aliquot out of the 55 µl total volume was analyzed. The percentage that was soluble or insoluble was determined by dividing the total cpm for either fraction by the total cpm in both fractions. The last column lists these percentages.

RIP Activity Assays

Protein Synthesis Inhibition Assay - Ambion's Untreated Rabbit Reticulocyte Lysate IVT Kit contains reticulocyte lysate and a master mix (- leucine), which contains the necessary components for translation, except leucine. By adding [^{14}C]-leucine to the above mixture and incubating at 30 °C, radioactively-labeled reticulocyte lysate proteins (mainly globin) are synthesized, as discussed under Methods. By setting up the reaction mixtures in the presence or absence of varying amounts of RIPs, the percentage of protein synthesis inhibition by the RIPs can be determined by comparing the amounts of radioactivity.

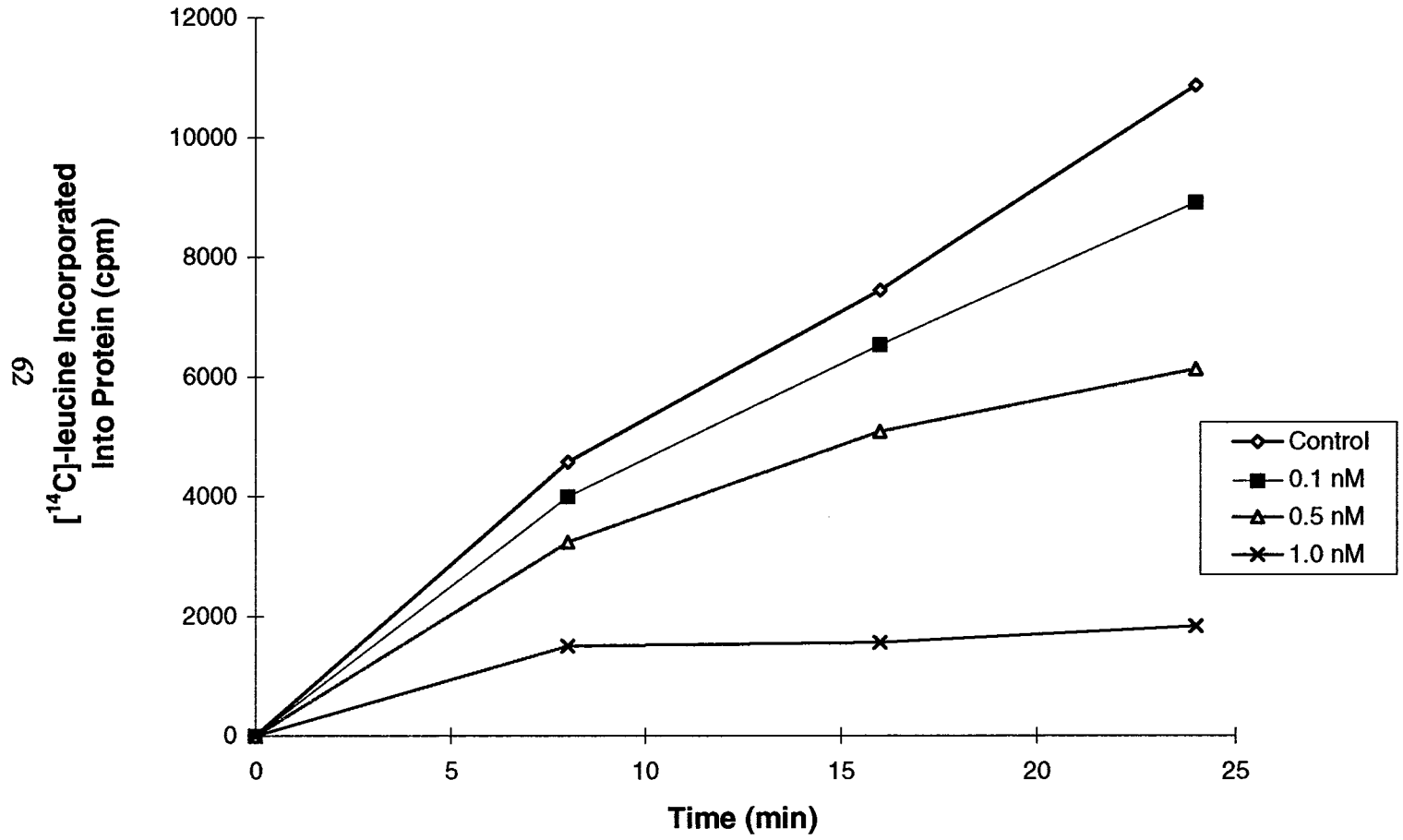
RIP-Specific N-Glycosidase Assay - In this assay, Promega's Untreated Rabbit Reticulocyte Lysate was used. As in the protein synthesis inhibition assay, the reticulocyte lysate is incubated with varying amounts of RIPs. However, in this assay, the RNA is extracted from the reticulocyte lysate and subjected to aniline treatment at acidic pH. Aniline treatment causes cleavage of the phosphodiester backbone at the site in the 28 S rRNA where depurination by the RIPs has occurred, as shown in Figure 1. Since this site of depurination in the S/R loop is approximately 390 (May *et al.*, 1989) to 460 (Morris *et al.*, 1992) nucleotides from the 3' end of the rabbit reticulocyte 28 S rRNA, two rRNA fragments are generated. The large fragment is over 4000 nucleotides in length and is referred to as the sub-28 S rRNA. The small 3' fragment is referred to as the Endo fragment or α-fragment. The electrophoretic mobility shift between the 28 S to sub-28 S rRNA can be detected by 3.5% PAGE, and the α-fragment can be detected by 3.5% - 5% PAGE, depending on the conditions (i.e., buffer, electrophoresis duration, and voltage).

Purified Gelonin-XOMA - The purified gelonin-XOMA was assayed for its ability to inhibit protein synthesis in rabbit reticulocyte lysate. Figure 12 shows the results using this RIP at various concentrations from 0.1 nM to 1.0 nM. The values

Figure 12

Protein Synthesis Inhibition of Purified Gelonin-XOMA

The results shown are from the protein synthesis inhibition assay in which the indicated concentrations of gelonin-XOMA (see legend) were added to the rabbit reticulocyte lysate mixture and incubated for 24 min. The control reaction is also shown; this reaction mixture is taken as the 100% value for protein synthesis (no inhibition). A 2 μl aliquot of each reaction mixture was used to measure the amount of [^{14}C]-leucine (radioactivity) incorporated into reticulocyte protein as described under Methods. The radioactivity is shown as counts per min (cpm) per 20 μl total reaction mixture.


12000
10000
8000
6000
4000
2000
0
0
5
10
15
20
25
Time (min)
[14C]-leucine Incorporated Into Protein (cpm)
Control
0.1 nM
0.5 nM
1.0 nM

represent the average of two experiments. The IC_{50} value, calculated at 16 min, was between 0.5 nM and 1.0 nM, but closer to 0.5 nM. The IC_{50} value for native gelonin isolated from *Gelonium multiflorum* is 0.4 nM (Stirpe *et al.*, 1992). So the *E. coli*-synthesized, purified gelonin-XOMA is about as active as native gelonin.

The purified gelonin-XOMA was then tested in the N-glycosidase assay at various concentrations from 0.1 nM - 1 μM. A copy of the UV photograph taken after ethidium bromide-staining of the polyacrylamide gel is shown in Figure 13. The α-fragment appeared in lane 3 when as little as 1.0 nM gelonin-XOMA was added to the reaction mixture. As the concentration of gelonin-XOMA increased, there was a pronounced increase in the amount of α-fragment produced, as seen in lanes 4 - 7. When 50 nM gelonin-XOMA was added to the reaction mixture, but the aniline treatment step was intentionally omitted, no α-fragment was produced, as seen in lane 1. When the gel was subsequently stained using Stains-all (results not shown), the α-fragments in lanes 3 - 7 were each as visible as when the gel was stained with ethidium bromide and viewed under UV light. This indicates that either method of visualizing the RNA was equally acceptable. No attempt to quantitate the amount of α-fragment in each lane was made.

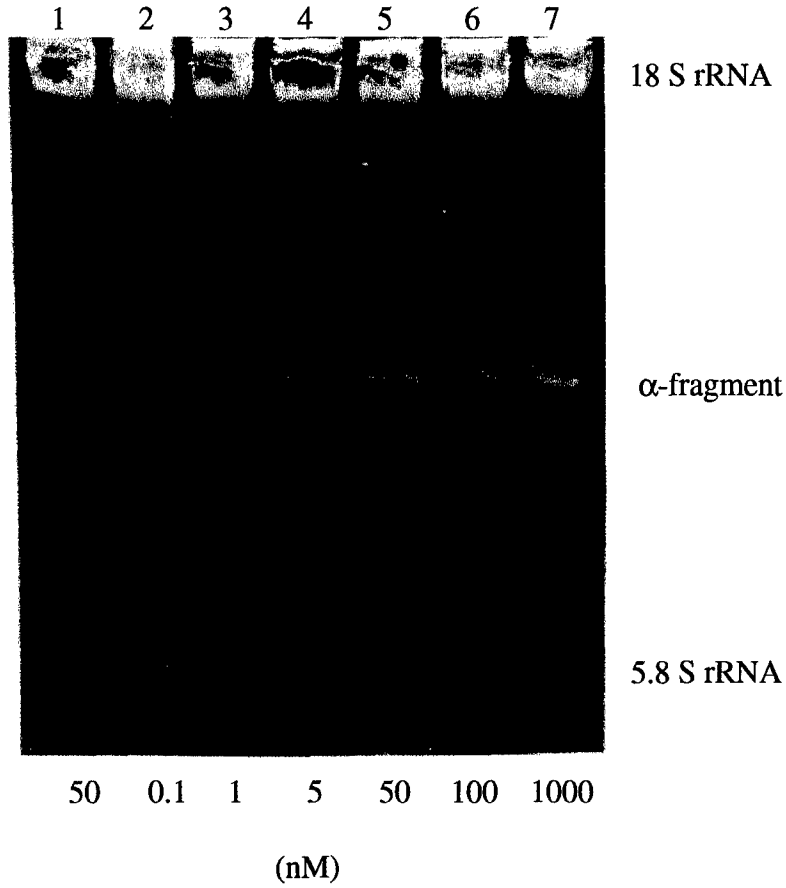
IMAC-Purified Thioredoxin-Gelonin - The high amounts of NaCl and imidazole present in the eluted thioredoxin-gelonin fractions were lowered by diluting and concentrating with 1X ribosomal buffer, as described under Methods. Thioredoxin-gelonin was tested for its ability to inhibit protein synthesis at 0.7 nM concentration. Figure 14 is a graph of the results from this assay. No inhibition of protein synthesis was observed compared to the control, which contained a buffer (20 mM Tris-HCl, pH 7.4, 0.2 mM NaCl, and 0.4 mM imidazole) with the same concentrations of imidazole and NaCl.

This experiment also supports what has been known for some time about the sensitivity of rabbit reticulocyte lysate to NaCl and imidazole. In one of the controls,

Figure 13

UV Photograph of the Polyacrylamide Gel -

N-Glycosidase Activity of Purified Gelonin-XOMA

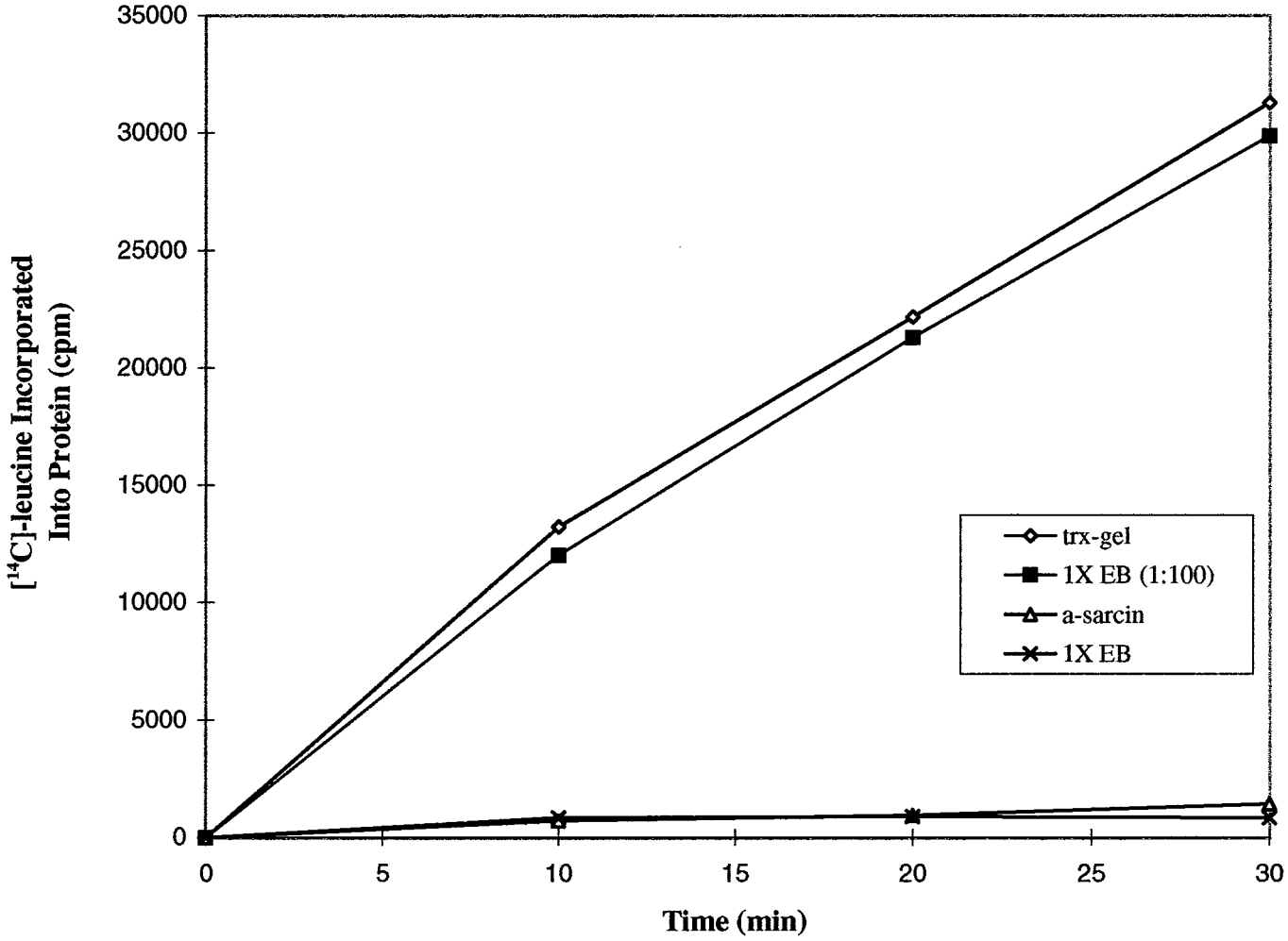


The UV photograph was taken from the ethidium bromide-stained polyacrylamide gel. The bands in each lane represent the extracted RNA from the reticulocyte lysate reaction mixtures containing the final concentrations of gelonin-XOMA indicated below each lane. The RNA in lane 1 was not treated with aniline; all other extracted RNA was subjected to aniline treatment at pH 4.6. The 28 S rRNA and sub-28 S rRNA fragments did not migrate into the gel, but the α-fragment (labeled to the right of the photograph) is clearly visible in lanes 3 - 7. Also labeled are the 18 S rRNA and the 5.8 S rRNA. All smaller RNAs were allowed to migrate off the gel.

Figure 14

Protein Synthesis Inhibition of IMAC-Purified Thioredoxin-Gelonin

The results shown are from the protein synthesis inhibition assay in which 0.7 nM IMAC-purified thioredoxin-gelonin was added to rabbit reticulocyte lysate and incubated for 30 min. Control reactions using 26 nM α-sarcin, 1X Elute Buffer (undiluted), and 1X Elute Buffer (1:100) are also shown. The reaction mixture containing the 1X Elute Buffer (1:100) is taken as the 100% value for protein synthesis (no inhibition); this mixture contained the same concentrations of NaCl and imidazole as the 0.7 nM IMAC-purified reaction mixture without any RIPs being present. [^{14}C]-leucine incorporation into reticulocyte protein (radioactivity) was measured as described under Methods and is shown as counts per min (cpm) per 20 µl total reaction mixture.


35000
30000
25000
20000
15000
10000
5000
0
0
5
10
15
20
25
30
[14C]-leucine Incorporated Into Protein (cpm)
Time (min)
trx-gel
1X EB (1:100)
a-sarcin
1X EB

1X Elute Buffer, which contains NaCl and imidazole, was added to final concentrations of 20 mM NaCl and 40 mM imidazole. Protein synthesis was inhibited to a greater extent than was observed in another reaction mixture containing 26 nM α-sarcin and no additional NaCl or imidazole. Protein synthesis was not inhibited when 1X EB (1:100) was used in the reaction mixture (when final concentrations of NaCl and imidazole were 0.2 mM and 0.4 mM, respectively).

Purification by IMAC (Denaturing Conditions) - The urea, imidazole, and some of the NaCl were removed from the eluted thioredoxin-gelonin fraction by diluting and concentrating with 1X N-glycosidase assay buffer, as described under Methods. Purified thioredoxin-gelonin was tested in the N-glycosidase assay at a final concentration of 1.3 μM. The RNA was extracted with and without aniline treatment. Neither the sub-28 S rRNA nor the α-fragment were present on the polyacrylamide gel after ethidium bromide-staining or Stains-all staining (results not shown).

Since thioredoxin-gelonin did not exhibit any N-glycosidase activity at 1.3 μM, the protein was not tested for activity in the protein synthesis inhibition assay. No attempt (other than the procedure described under Methods for diluting and concentrating the protein with 1X N-glycosidase assay buffer) was made to renature this protein. Recall that the initial purpose of fusing gelonin to thioredoxin was to be able to synthesize a soluble product. Denaturing and then attempting to renature thioredoxin-gelonin from inclusion bodies defeats the initial purpose of this research. Why not just try to denature and renature the overexpressed 29 kDa gelonin from inclusion bodies?

rEK-Cleaved Gelonin - The products from the rEK cleavage reaction were tested in the N-glycosidase assay at a final concentration of 76 nM gelonin, but exhibited no activity (results not shown). The rEK cleavage products were not tested in the protein synthesis inhibition assay.

E. coli Lysates Containing the Overexpressed RIPs - Thioredoxin-gelonin was found to be very labile to proteolysis (even with buffers containing several protease inhibitors, as described under Methods) especially during the time period between lysing the cells and purifying this protein by IMAC under non-denaturing conditions. So it was thought that it would be best to test for enzymatic activity immediately after lysing the cells (without purifying the protein). The lysates from induced *E. coli* BL-21(DE3)pLysS cells containing either pET-21a/gel, pET-32a/gel or pET-32a were immediately tested in the protein synthesis inhibition assay. The lysate from cells containing the pET-32a plasmid were used as a control since this plasmid contains the genetic information to overexpress only thioredoxin plus the linker sequence. This lysate contains the same *E. coli* ions and macromolecules (minus the RIPs) as the other two lysates.

Figure 15 is a graph of the results from this assay. At final concentrations of 100 nM gelonin or 2 μM thioredoxin-gelonin, no protein synthesis inhibition was observed beyond that of the lysate from the control cells. Due to the inability of either lysate to inhibit protein synthesis at such high RIP concentrations, coupled with the presence of *E.coli* ribonucleases (RNases), the lysates were not tested in the N-glycosidase assay.

E. coli-Overexpressed, Soluble RIPs - Lysates, as described above, were centrifuged to separate the soluble proteins from the insoluble proteins, as discussed under Methods. The soluble fractions containing the overexpressed RIPs were assayed for N-glycosidase activity in the rRNA cleavage assay. The RNA was extracted and subjected to aniline treatment. A copy of the UV photograph taken after ethidium bromide-staining of the polyacrylamide gel is shown in Figure 16. Lanes 4 and 5 show the extracted RNA from the reaction mixtures containing 5 μM thioredoxin-gelonin and 2 μM gelonin, respectively. The α-fragment is not present in either of these lanes, but is clearly visible in lane 1 in which 2.9 μM α-sarcin was

Figure 15

Protein Synthesis Inhibition of the Cell Lysates

The results shown are from the protein synthesis inhibition assay in which the *E. coli* lysates containing overexpressed RIPs were added to rabbit reticulocyte lysate and incubated as described under Methods. Each reaction mixture contained final concentrations of either 100 nM gelonin (labeled "100 nM gel"), 2 μM thioredoxin-gelonin (labeled "2 μM trx-gel"), or overexpressed thioredoxin alone (labeled "trx-control"). In a separate reaction mixture, an equal volume of water (as *E. coli* lysate) was added instead of any *E. coli* lysate. This last reaction mixture (labeled "water") was taken as 100% protein synthesis (no inhibition). It was used an additional control in order to estimate the extent of protein synthesis inhibition due to factors present in the *E. coli* lysate (i.e., inorganic ions, macromolecules, RNases). [^{14}C]-leucine incorporation into reticulocyte protein (radioactivity) was measured as described under Methods and recorded as counts per min (cpm) per 20 μl total reaction mixture.

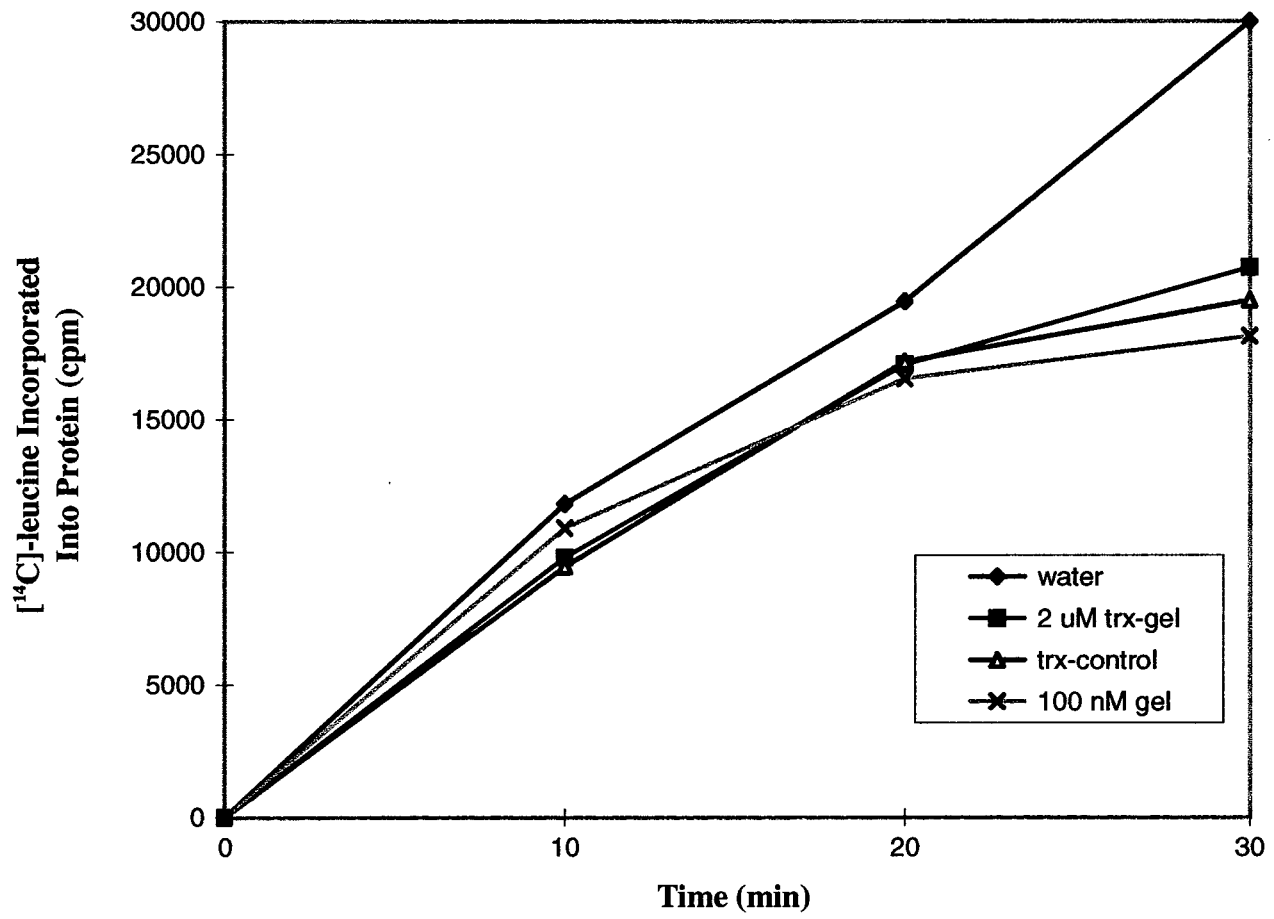
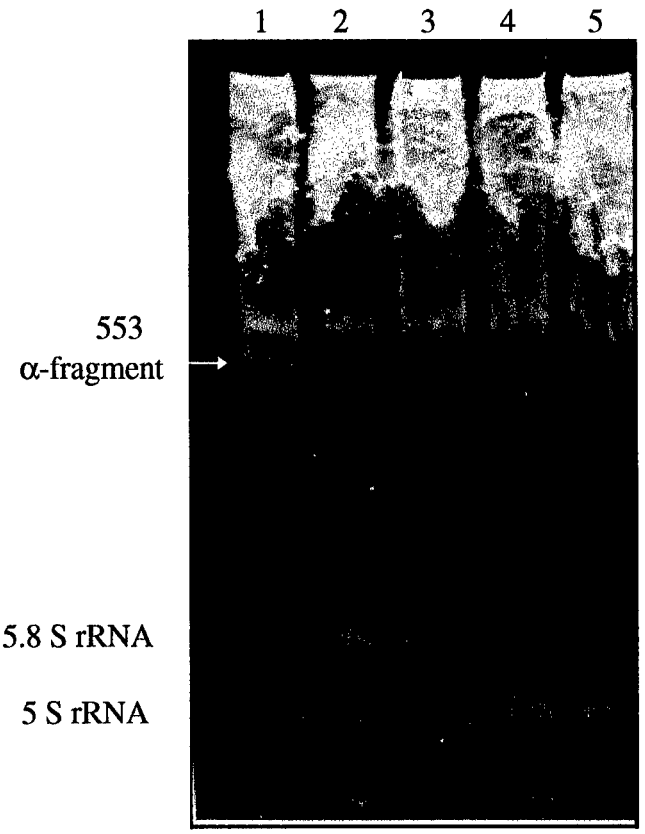

30000
25000
20000
15000
10000
5000
0
0
10
20
30
[14C]-leucine Incorporated Into Protein (cpm)
Time (min)
water
2 uM trx-gel
trx-control
100 nM gel

Figure 16

UV Photograph of the Polyacrylamide Gel -

N-Glycosidase Activity of the Soluble Fractions



The UV photograph taken from the ethidium bromide-stained polyacrylamide gel. The bands in each lane (labeled to the left of the photograph) represent the RNA extracted from the reticulocyte lysate reaction mixtures after incubation with the following final concentrations of RIPs: (lane 1) 2.9 μM α-sarcin, (lane 2) *in vitro*-synthesized, soluble fraction containing 9 nM thioredoxin-gelonin, (lane 3) *in vitro*-synthesized, soluble fraction containing 12.6 nM gelonin, (lane 4) *E. coli*-overexpressed, soluble fraction containing 5 μM thioredoxin-gelonin, (lane 5) *E. coli*-overexpressed, soluble fraction containing 2 μM gelonin, The RNA extracted from all reaction mixtures was subjected to aniline treatment at pH 4.6. The α-fragment appears in lane 1 only.

added to a separate reaction mixture as a positive control. Any attempts at testing a larger volume of the soluble fractions, or more concentrated amounts of the soluble proteins, led to degredation of the rRNA (most likely due to *E. coli* RNases). Lanes 2 and 3 show the extracted RNA from reactions mixtures containing the soluble gelonin and thioredoxin-gelonin fractions synthesized by *in vitro* coupled transcription/translation, as described below.

The *E. coli*-overexpressed, soluble RIPs were not tested in the protein synthesis inhibition assay due to the lack of activity of these two proteins in the N-glycosidase assay. Additionally, too many other factors (low pH, inorganic ions, RNases,) were present in these fractions that could potentially affect the amount of protein synthesis in the reticulocyte lysate.

In Vitro-Synthesized, Soluble RIPs - The remaining *in vitro* coupled transcription/translation reaction mixtures were centrifuged to separate the soluble proteins from the insoluble proteins (full-length, insoluble RIPs and less-than-full-length, ribosome-bound RIPs). The amount of each RIP that was soluble and insoluble was measured as discussed under Methods.

The unpurified, soluble RIPs were immediately tested in the N-glycosidase assay at final concentrations of 9 nM thioredoxin-gelonin and 12.6 nM gelonin. The RNA was extracted and subjected to aniline treatment, pH 4.6. A copy of the UV photograph taken after ethidium bromide-staining of the polyacrylamide gel is shown in Figure 16. Lanes 2 and 3 show the RNA products from the reaction mixtures containing the *in vitro*-synthesized, soluble thioredoxin-gelonin and gelonin fractions, respectively. The α-fragment is not present in either lane, but is clearly visible in lane 1 in which 2.9 μM α-sarcin was added to a separate reaction mixture as a positive control.

Although the amount of α-sarcin used appears high in comparison to the amount of thioredoxin-gelonin and gelonin used, the α-fragment was clearly visible in

another experiment when only 1 nM gelonin-XOMA was added to the reaction mixture, as seen in Figure 13, lane 3. Therefore, it was concluded that neither of the *in vitro*-synthesized, soluble RIPs showed any N-glycosidase activity at 12.6 and 9-fold higher concentrations than was necessary to see activity with the purified gelonin-XOMA.

In a separate experiment, both soluble fractions were tested in the protein synthesis inhibition assay at final concentrations of 5.5 nM gelonin and 26 nM thioredoxin-gelonin. For the control, an equal volume of soluble fraction from an *in vitro* coupled transcription/translation reaction mixture containing no plasmid was used. There was no inhibition of protein synthesis at these concentrations in comparison with the control reaction mixture (results not shown).

Discussion

Comparison of the Assays Used

The protein synthesis inhibition assay is much faster and easier to perform than the N-glycosidase assay. It also allows the researcher to make quantitative comparisons about the activity of RIPs by comparing IC_{50} values. This would also be useful when comparing mutant RIPs against the wild-type RIP. The N-glycosidase assay is not quantitative. The protein synthesis inhibition assay was found to be approximately one order of magnitude more sensitive than the N-glycosidase assay; Figure 12 indicated that even some inhibition of protein synthesis was observed at 0.1 nM gelonin-XOMA, whereas the α-fragment did not appear in Figure 13 until 1.0 nM gelonin-XOMA was used in the reaction mixture. This difference in sensitivity may in part be due to the inevitable loss of RNA during extraction and aniline treatment.

The weakness of the protein synthesis inhibition assay is that the reticulocyte lysate is very sensitive to experimental conditions (i.e., pH, temperature, and inorganic ion concentrations). The results from one experiment (shown in Figure 14) indicated that 20 mM NaCl and 40 mM imidazole inhibited protein synthesis to a greater extent than 26 μM α-sarcin. This emphasizes the fact that this assay lacks the specificity of the N-glycosidase assay because many factors (through interactions with ribosomal or ribosomal-associated proteins, aminoacyl tRNA synthetases) can cause inhibition of protein synthesis in reticulocyte lysate. An observed inhibition of protein synthesis does not necessarily indicate that the specific N-glycosidase activity of the RIP inactivated the ribosome. Therefore, both assays should be performed when assessing the enzymatic activity of RIPs.

Inactivity of Gelonin and Thioredoxin-Gelonin

Table 3 is a summary of the concentrations at which the various gelonin and thioredoxin-gelonin fractions were assayed for activity. Neither of these two proteins showed any activity in the protein synthesis inhibition assay or the N-glycosidase assay at any of the concentrations tested. The purified gelonin-XOMA exhibited an IC_{50} value of approximately 0.5 nM in the protein synthesis inhibition assay (Figure 12), and exhibited RIP-specific N-glycosidase activity at 1.0 nM final concentration (Figure 13). Therefore, it was concluded that neither *in vitro* nor *in vivo* synthesis of either of these proteins from the pET-21a/gel or pET-32a/gel plasmids leads to the formation of enzymatically-active RIPs. Some possible explanations for the lack of activity and some areas for future research are discussed, after comparing the solubility of gelonin and thioredoxin-gelonin.

Comparison of the Solubility of the RIPs

From Figure 10, it was evident that induction of the two *E. coli* BL-21(DE3)pLysS colonies containing the pET-32a/gel plasmid did not result in a higher percentage of soluble thioredoxin-gelonin protein compared to the amount of soluble gelonin from the induction of two *E. coli* BL-21(DE3)pLysS colonies containing the pET-21a/gel plasmid. Therefore, it was concluded that fusion to thioredoxin did not increase the solubility of gelonin when synthesized *in vivo*, at least in this design of thioredoxin-gelonin (with a 54 amino acid-linker sequence separating thioredoxin from gelonin).

The results shown in Figure 11 and Table 2 indicated that 71% of the thioredoxin-gelonin synthesized by *in vitro* coupled transcription/translation was soluble, whereas only 22% of the gelonin was soluble. Therefore, this indicates that

Table 3

Summary of the Results on Enzymatic Activity of the RIPs

Fraction	Protein Synthesis Inhibition Assay	rRNA Cleavage Assay	Remarks
E. coli lysate	*100 nM gel, 2 μM trx-gel	Not tested	*Figure 15
E. coli soluble fraction	Not tested	*2 μM gel, 5 μM trx-gel	*Figure 16
E. coli IMAC-purified (Non-Denaturing)	*0.7 nM trx-gel	Not tested	*Figure 14
E. *coli* IMAC-purified (Denaturing)	Not tested	1.3 μM trx-gel (Not Shown)	
rEK-cleaved gel	Not tested	76 nM (Not Shown)	
In vitro-synthesized soluble fractions	5.5 nM gel, 26 nM trx-gel (Not Shown)	*12.6 nM gel, 9nM trx-gel	*Figure 16

For each of the various fractions listed above, the synthesized RIPs were assayed for their ability to inhibit protein synthesis in rabbit reticulocyte lysate and/or their specific N-glycosidase activity on rabbit reticulocyte ribosomes. The concentrations shown are the final concentrations of the RIPs in the reaction mixtures. Gelonin is abbreviated "gel" and thioredoxin-gelonin is abbreviated "trx-gel". The Remarks column lists the figures which show these results. No activity was observed at any of these final concentrations in either assay, whereas the purified gelonin-XOMA inhibited protein synthesis with an IC_{50} value of approximately 0.5 nM and exhibited observable RIP-specific N-glycosidase activity at 1.0 nM.

fusion to thioredoxin did increase the solubility of gelonin when synthesized by the *in vitro* coupled transcription/translation procedure developed by Zubay and refined by Kudlicki (Kudlicki *et al.*, 1992).

There was a large increase in the percentage of soluble thioredoxin-gelonin when synthesized by this *in vitro* system compared to the percentage of soluble thioredoxin-gelonin when overexpressed in *E. coli*. There was also a very large percentage of soluble thioredoxin-gelonin (71%) compared to the amount of soluble gelonin (22%) when synthesized by this *in vitro* system. These results could indicate that other eukaryotic proteins that are insoluble when synthesized in *E. coli* could be synthesized in soluble form by this *in vitro* coupled transcription/translation system after fusion of the gene to *trxA*. If this were true, this system would have important implications for the biotechnology industry and for research.

Possible Effects of the Differences in Primary Amino Acid Sequences on the Solubility and Enzymatic Activity

As stated under Introduction, gelonin can be assigned to the $\alpha + \beta$ class of proteins because its N-terminal region (the first 100 amino acids) has predominantly β secondary structure, while the remainder of the protein has predominantly helical structure, based on the X-ray structure of native gelonin (Hosur *et al.*, 1995).

One of the major differences between gelonin-MDA and gelonin-XOMA is the presence of an 8 amino acid peptide in gelonin-MDA between Lys_{103} and Thr_{112}, which does not exist between Lys_{104} and Thr_{105} of native gelonin or gelonin-XOMA (see Figure 5). Based on its position in the primary amino acid sequence, this 8 amino acid peptide would most likely lie directly between the α and β regions. It is difficult to predict what effects this might have on the tertiary structure and hence the enzymatic activity of gelonin-MDA, since the crystal structure of gelonin-MDA has not been determined. However, these 8 amino acids would be very close in the primary

sequence to some important residues that were cited as being part of the active site of native gelonin (i.e., Gly_{111} and Tyr_{113}) or involved in maintaining contacts between different regions of secondary structures (i.e., Leu_{107} and His_{108}). These 8 amino acids in gelonin-MDA could shift residues out of position in comparison to native gelonin so that they are no longer contributing factors at the active site or are no longer involved in maintaining critical contacts. They could also be responsible for the inability of the 29 kDa gelonin-MDA to fold properly, as demonstrated by the large percentage of this protein that is insoluble when overexpressed in *E. coli* and when synthesized by *in vitro* coupled transcription/translation. Removal of these 8 amino acids in gelonin-MDA (removal of the 8 codons in the synthetic gelonin-MDA gene) was not the focus of this research and therefore was not attempted. However, this would be an interesting future experiment, and could possibly result in the ability to synthesize gelonin-MDA in *E. coli* or by *in vitro* coupled transcription/translation.

The other major difference between gelonin-MDA and gelonin-XOMA is the presence of a disulfide bond in gelonin-XOMA. The XOMA group engineered several recombinant gelonin-XOMA analogs, each containing only one of the two cysteine residues, and most were reportedly active *in vitro* (Better *et al.*, 1994). Therefore, this single, intrachain disulfide bond is probably not significant for the proper folding or N-glycosidase activity of gelonin. However, it is important to note that both the XOMA and MDA research groups used only one of two important assays to determine if their proteins were active: the protein synthesis inhibition assay. Neither group used the RIP-specific N-glycosidase assay.

It is important to note that the MDA authors (Rosenblum *et al.*, 1995) discussed that during the purification of native gelonin from *Gelonium multiflorum*, a contaminating protein was present. Perhaps this protein was also present during the proteolytic cleavage and subsequent Edman degredation. Although this is only a hypothesis, it is possible that the 8 amino acids found during Edman degredation, but

not found during cDNA analysis after reverse transcription of the mRNA isolated from *Gelonium multiflorum* (Nolan *et al.*, 1993), belong to this contaminating protein and not to native gelonin.

The Possible Effects of the Linker Region

The original thioredoxin-fusion plasmid, pTRXFUS, designed by LaVallie contained only 10 codons (coding for the oligopeptide GSGSGDDDDK) between thioredoxin and the multiple cloning region (LaVallie *et al.*, 1993). In comparison, Novagen's pET-32a plasmid may be poorly designed; its linker region contains a His-Tag sequence, a thrombin cleavage site, an S-Tag sequence, and an enterokinase cleavage site (see Figure 5). Even if the most upstream restriction site (NcoI) in the multiple cloning region is used for the cloning of a particular insert, there are still 51 amino acids in the linker region. The second most upstream restriction enzyme site (EcoRV) was the site used in this research for molecular cloning, and the linker peptide contains 54 amino acids. Since the amino acids in this large linker region are covalently attached and in such close proximity to the gelonin-MDA during synthesis, they may interfere with the proper folding or contribute to the improper folding of the C-terminal gelonin-MDA. The use of a smaller linker (similar to the one in LaVallie's pTRXFUS plasmid) probably would have increased the potential for the gelonin-MDA to remain soluble, thus increasing its potential to fold correctly. This is only a hypothesis and may not be the correct interpretation.

On the contrary, the linker sequence may not have been the cause of gelonin's lack of activity when fused to thioredoxin. The 29 kDa gelonin-MDA from pET-21a/gel formed inclusion bodies in *E. coli* even in the absence of fusion to any other proteins, as discussed under Introduction. This was the reason for fusing gelonin to thioredoxin and the basis for this research (to try to prevent the aggregation of gelonin

and increase its potential to fold correctly). Fusion of the gene for another RIP (one that has been shown to form an active product when synthesized in bacteria, such as ricin A-chain), to the same EcoRV site in pET-32a might be a good control to see if the linker region is interfering with the C-terminal RIPs' ability to fold properly. If the thioredoxin-ricin A-chain fusion protein is synthesized in soluble form and exhibits enzymatic activity, then this would suggest that perhaps the large linker sequence is not what caused thioredoxin-gelonin to form inclusion bodies when overexpressed in *E. coli*, or its lack of activity when synthesized by *in vitro* coupled transcription/ translation (and its lack of activity when overexpressed in *E. coli*).

Another interesting future study would be to remove most of this large linker region (the DNA region coding for the His-Tag, S-Tag, and thrombin and enterokinase cleavage sites) and determine what effects this would have on the solubility and enzymatic activity of gelonin-MDA. One way this could be accomplished is as follows. The gelonin-MDA gene was inserted into the pET-21a plasmid at the NdeI restriction enzyme site at the 5' end of the gene; this NdeI site also contains the start site for the synthesis of gelonin-MDA. In the pET-32a plasmid, there are two NdeI sites flanking *trxA* and the DNA sequence coding for the first 5 amino acids (GSGSG) of the linker region (see Figure 4). The first NdeI site in pET-32a contains the start site for the synthesis of thioredoxin. By digesting pET-32a with NdeI, *trxA* and part of the linker can be isolated and then inserted into pET-21a/gel at it's only NdeI site. Theoretically, 50% of the *trxA* inserts should be correctly oriented when cloned into the pET-21a/gel plasmid. This would yield a thioredoxin-gelonin fusion protein separated by only a small linker region without any method to separate gelonin from thioredoxin. However, the solubility and enzymatic activity of the fusion protein could be tested.

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This thesis was typed by the author.